BIOLOGICAL REVIEWS

AND

BIOLOGICAL PROCEEDINGS

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CONTENTS

INDEX OF AUTHORS

GROWTH FACTORS OF LOWER ORGANISMS

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CONTENTS.

I. INTRODUCTION.

So far as the author is aware, the only article in which any attempt has been made to cover the whole subject of growth factors of lower organisms is that of Sergent (1928). In it the factors are classified according to their origin. As the following article is written mainly from the point of view of the organisms concerned, that of Sergent is valuable as a cross-reference, especially as it is well indexed, although on account of its wide scope it is not very exhaustive. A brief outline of the subject will

be found in the Medical Research Council's *System of Bacteriology* (1930), p. 252. The published work dealing with the growth factors of lower organisms has become so extensive that it is impossible to review it all completely in the space allotted. Even if it were possible so to condense the literature, the resulting article would be too stilted to be intelligible, and the enumeration of all the conflicting opinions would only add to the confusion. In an endeavour to avoid this, the author has attempted to cover the whole subject in a general manner. For the sake of convenience the subject has been divided into four main sections, each, with the exception of the first, consisting of two parts:

(1) A brief summary including, where possible, reference to some published "key" article which reviews the subject in greater detail (such "key" articles are marked in the list of references with an asterisk).

(2) A critical discussion of the "key" article and of any work or points of view that are not, in the author's opinion, adequately dealt with therein.

To save unnecessary repetition and to avoid adding to the ever-swelling volume of scientific literature, any work which has been adequately dealt with in the "key" article receives little mention here. As regards nomenclature, in Section III every effort has been made to follow the classification suggested by the Society of American Bacteriologists and the older names are given in parentheses. In other sections it has been thought wisest in some cases to adhere to the particular nomenclature used in the original articles to which reference is made.

The subject of growth factors for lower organisms needs no special introduction. In addition to the major nutrient foodstuffs supplying energy to bacteria, it has become well recognised that certain species at least, require the presence of traces of other materials—accessory food factors—in order that they may develop. These factors form the subject-matter for the greater part of the present article (Section III). While it is highly desirable that the term "growth factor" should be restricted to those factors which are essential, it has been necessary to include growth stimulants the indispensable nature of which remains as yet unproven. In addition there are various other factors with which the bacteriologist is well acquainted. The most important of these are considered briefly in Section II. The remaining sections deal with the relation of growth factors to vitamins (IV) and the production of growth factors by lower organisms (V). There is, of necessity, a considerable amount of overlapping between the sections (*e.g.* between III and IV), but for the sake of simplicity the number of cross-references has been reduced to a minimum.

The author wishes to acknowledge his indebtedness to his colleagues at Oxford and at Reading for their most valuable criticism and advice, and to his wife for her generous assistance in the preparation of the manuscript.

II. EXPERIMENTAL PROCEDURE.

The importance of details of technique in experimental studies of growth factors has been emphasised from time to time by various workers, *e.g.* in articles such as that on the "Influenza Group of Bacteria" in the Medical Research Council's

System of Bacteriology (1929), but it has often been neglected. The following special points may be mentioned, as their importance cannot be over-emphasised.

A. Factors connected with the medium.

(1) *Inhibitory factors.*

Numerous substances are known which markedly inhibit growth, *e.g.* salts of heavy metals. These may be present in the media which are used for testing growth factors. The only remedy that has been suggested is to include in all tests a control culture containing a relatively high concentration of growth stimulants in which, it is hoped, any inhibitory effects will be detected, *e.g.* Orr-Ewing and Reader (1928 *a*). Such a precaution is necessarily rather crude and may not detect inhibitory agents whose effect may not be evident at high concentrations of growth factors but may be quite sufficient to interfere with the activity of lower concentrations, such as are usually employed in the test cultures. Moreover, it is known that in some cases excess of a growth stimulant may inhibit growth, *e.g.* Vansteenberge (1917), Fulmer, Duecker and Nelson (1924). The problem of inhibitory substances is one of the greatest difficulties that beset the experimenter.

(2) *Sterilisation of the medium.*

Another difficulty is presented in the method of sterilisation of the medium. Heat may have marked effects on the growth-promoting factors, *e.g.* destruction, or it may even cause production of stimulatory substances, as recorded by Fulmer and Huesselmann (1927), Fulmer, Williams and Werkman (1931), or of inhibitory substances, as noted by Lewis (1930). Peskett and O'Brien (1930) found some samples of cotton-wool to contain growth factors for yeast, and observed that in some cases where wetting of the wool plugs occurred during sterilisation, the medium was enriched in growth factors. Sterilisation by filtration is frequently reported as causing serious losses of growth factors on account of adsorption or inactivation at the surface of the filter.

(3) *Surface tension.*

The question of the importance of surface tension as a factor in the growth of lower organisms has often been mentioned in the literature, but there are few papers devoted to the subject. An article by Reader (1927 *b*) refers to some of the earlier work, which had shown that there was some evidence of a correlation between the amount of growth and the surface tension of the medium. Reader's results indicate that in the case of *Streptothrix corallinus*, the amount of growth produced in media containing additions of broth or antineuritic vitamin is not related to change in surface tension. Correlation was, however, noted between the type of growth (film or deposit) and change in interfacial tension of the medium against benzene. At about the same time a paper by Pizarro (1927) appeared, confirming the earlier work of Gibbs, Batchelor and Sickels (1926), showing that there was no correlation between the amount of growth and the surface tension.

In many cases a marked inhibition of growth was observed which could be better attributed to the chemical nature of the depressants used than to the altered surface tension. This paper contains several references not cited by Reader, and, as the latter author points out, experiments involving addition of substances such as soaps are beyond the scope of her paper. Both agree that there is no indication that pellicle formation is suppressed at lowered surface tension. Surface tension thus appears to be of very little importance as a factor in determining the amount of growth, though it may play a part in regulating the type of growth (film or deposit). The reader should consult Buchanan and Fulmer (1928, pp. 170, 448, 455). Undoubtedly, as Larson (1928) says, "The nutritive material in culture media must be concentrated at the bacteria-water interfaces," and no one will deny the importance of surface in biological phenomena. Concentration at an interface is, however, a general property of all solutes which lower surface tension. Thus, for example, excretory, toxic or inhibitory substances may likewise be concentrated. This must be borne in mind before any sweeping conclusions are drawn as to the significance of surface tension in any particular phase of biological activity (see Section III, *Neisseria gonorrhoeae*).

For a discussion of the effects of bile and bile salts on growth, the reader is referred to Buchanan and Fulmer (1930, p. 537).

(4) *Carbon dioxide.*

Recently Wilson (1931) has shown that *Brucella abortus* (bovine) requires carbon dioxide for growth. He suggests that it acts as a specific cell-wall penetrant which lowers the intracellular pH. According to the earlier work of Rockwell and Highberger (1927), carbon dioxide is necessary for the growth of all bacteria, yeasts and moulds. With the exceptions cited by these authors, very few publications on growth factors mention this subject. It is of some importance, as the conditions under which inhibition of growth occurs (owing to lack of carbon dioxide) are similar to those of the control cultures which are used in testing growth factors, *e.g.* the use of small inocula and a "lean" medium.

(5) *Hydrogen-ion concentration.*

Apart from the intrinsic importance of pH, it must be noted that the growth-accelerating effect of accessory substances may be optimal when the pH is far removed from the optimum value for growth in ordinary media. This effect was observed by Shwartzman (1924 *a*) in the case of the growth of *B. dysenteriae* (Shiga) in the presence of extract of tomato. The best growth-promoting effect occurred at pH 8·2–8·6, a range which is too alkaline for growth in the absence of tomato extract. The phenomenon was found to be due in part to the fact that at lower pH values, the cultures reached the inhibitory point more rapidly (pH 4·7, Cohen and Clark, 1919). At the same time, the critical point at which inhibition occurred was shifted towards the alkaline side to pH 5·4–5·6 by the presence of tomato extract, perhaps on account of the organic acids contained therein (Shwartzman, 1924 *b*).

Shwartzman (1924 c) also noted a complete disappearance of the growth factors from cultures as soon as the pH reached a value of 5·2. This effect was ascribed to the production of unknown factors which inactivate the growth factors (at pH 5·2), and are produced by actively multiplying bacteria. In addition to *B. dysenteriae* (Shiga), the following organisms were studied: *B. dysenteriae* (Flexner), *Brucella abortus*, *Pneumococcus* type 2, *B. typhosus* and *Streptococcus viridans*.

(6) *Oxidation-reduction potential.*

Recently the oxidation-reduction systems of cells have been extensively studied, and numerous publications have appeared dealing with measurements of the oxidation-reduction potentials of bacterial cultures at various stages in their growth. The full significance of such observations is not yet apparent, and discussion of them is beyond the scope of this review. The reader is referred to Jordan and Falk (1928, chap. 12), Clark (1928), and Hewitt (1931).

(7) *Amino acids.*

A comprehensive study of this subject has been made by Koser and Rettger (1919). Their paper contains a valuable summary of the conclusions of earlier workers in addition to their own results and is too detailed to be dealt with satisfactorily here. The articles by Berman and Rettger (1918), M'Leod and Wyon (1921), Mueller (1921 a and b, 1922 a and b, and 1923), Wyon (1923), Hosoya and Kuroya (1923 a), Whitehead (1924), Gordon and M'Leod (1926), M'Leod, Wheatley and Phelon (1927) and Sergent (1928, p. 79) should also be consulted.

(8) *Salt ratios.*

There are very few cases reported in the literature in which the most suitable composition of the basal medium has been determined thoroughly. To cite examples, Fulmer, Nelson and Sherwood (1921) studied the salt requirements of a species of yeast and observed that the optimum concentration of ammonium salts varied with the temperature. Reader (1927 a) made a thorough investigation of the salt requirements of *Streptothrix corallinus*, a white *Streptothrix* and *Sarcina aurantiaca*. These articles alone are sufficient to indicate the expediency of making careful investigation of the basal salt requirements of an organism before attempting to study its less definite "growth factors."

B. FACTORS CONNECTED WITH THE ORGANISM.

(1) *Size of inoculum.*

Large inocula may carry with them possible sources of growth factors which are uncontrolled by the investigator, or some of the inoculated material may die, and provide utilisable food material. In carefully controlled experiments, the inoculum should be washed, a precaution which seems rarely to have been taken, and minimal inocula should be used. The inoculum should not be too small, however, as Wilson (1922) has shown (for *Bact. suipestifer*) that even during the

logarithmic phase of growth seldom more than 90 per cent. of the organisms are viable. Since the growth of an organism must occur in immediate proximity to the inoculum, it is very doubtful whether the use of solidified media, which Kollath (1926) advocates in preference to liquid, would materially check diffusion of growth factors that may be introduced with the seeding, though it is true that the spread of bacteriophage, for example, is small on agar. In the case of the gonococcus, Cole and Lloyd (1917) emphasise the importance of using small seedings (see also Lloyd, 1916).

The use of large inocula introduces another factor, the so-called "allelocatalytic" effect according to T. B. Robertson (1921, 1924). A similar type of phenomenon, called in this case "communal activity," was noted by Churchman and Kahn (1921) in studying the behaviour of bacterial cells towards gentian violet. Robertson observed that the cells mutually accelerated each other's growth by production of a factor "x." This has been denied by Cutler and Crump (1923). In the case of yeasts, Lindner (1905), Henneberg (1907) and others have claimed that a single cell may benefit from the presence of other cells (see also Rubner, 1913), though Clark (1922) reported that "crowding" had little effect. The latter conclusion has been confirmed by Peskett (1925, 1928 a); a similar conclusion was reached by Ahuja (1929) in the case of *B. typhosus*.

(2) *Characteristics of the organism.*

The ability of many organisms to change their characteristics on repeated sub-culture is so well known that it need only be mentioned here. Among other attributes the need for growth factors has been observed to decrease on repeated subculture, *e.g.* in the case of the meningococcus by Lloyd (1916) and in the case of yeast, by Pringsheim (1906), though this was stoutly denied by Ide (1907). The problem is complicated by the fact that closely allied species of yeast, for example, exhibit wide variations in their ability to develop on a synthetic medium (Devereux and Tanner, 1927), a fact which is sometimes overlooked. Similarly, Sergent (1928, p. 98) noted disparity between the growth responses of three strains of the gono-coccus, but no data are given as to the distinctness of the strains used in these experiments.

Thus it is not surprising to find that widely separated species contrast noticeably in their need for, and responses to, growth factors. Bacteria have been classified into three groups according to their need for growth factors by Hosoya and Kuroya (1923 a) and Sergent (1928, p. 12). See also Section III, Summary.

III. GROWTH FACTORS REQUIRED BY LOWER ORGANISMS.

SUMMARY.

(a) *Bacteria (Schizomycetes).*

One of the characteristics of the members of the Nitrobacteriaceae is that they are capable of growth in media of known composition, securing their energy by direct oxidation of simple compounds of carbon, hydrogen, and nitrogen. For this

reason they have been used to some extent for studies of their powers of syn-
thesising growth factors (see Section V). Their need for specific growth factors
can be dealt with very briefly as it is practically nil, although numerous substances
such as auximones (see Stephenson, 1930, chaps. 8 and 9) may stimulate their
growth.

There is evidence for the need of accessory growth factors in the case of many
members of the Coccaceae, though much of it is so fragmentary that no coherent
review is possible. Wyon (1923) noted differences in the growth requirements of
Neisseria intracellularis (*Meningococcus*) and *Diplococcus pneumoniae* (*Pneumococcus*),
but definite knowledge of the factors required by the latter type of organism is,
as yet, very meagre. In the case of the haemolytic streptococci two factors have
been fairly definitely established, one of which may be associated with blood (*vide
infra*). As regards the other members of the Streptococceae the evidence for the
need of growth factors has not been clearly defined, and remains unsatisfactory.
The work on *Neisseria gonorrhoeae* (*Gonococcus*) has been briefly summarised in the
Medical Research Council's *System of Bacteriology* (1929, p. 248). At least two
factors and possibly a third are required, but their "precise characteristics are
difficult to define." Some theories have been advanced as to their mode of action,
but such theories have little experimental support. Knorr (1925 *a*) has reviewed
the work that has been done on many of the organisms of this family and in parti-
cular his section on *N. intracellularis* ("*Meningococcus*," p. 660) may be mentioned.
In this case there is definite evidence for the need of at least one factor, and some
support for the view that its mode of action is to increase proteolysis. The simpler
growth requirements of *N. intracellularis* agree with the fact that it is easier to
cultivate than *N. gonorrhoeae*. The work that has been done on the staphylococci is
mentioned in Section IV. No results seem to have been obtained which clear up any
possible interrelationships that may occur between the various factors mentioned
above.

In the Bacteriaceae, Sanborn (*vide infra*) has described stimulating effects in the
growth of a species of Cellulomonas which appear to be due to an essential substance
in the extracts of various materials which he employed. Werkman (1927) pointed
out that the stimulation may have been due only to the supply of available nitrogen
or to the sources of energy in Sanborn's extracts. More attention has been directed
to the growth requirements of one of the Haemophileae than to those of any other
bacteria. For a long time past the presence of blood has been thought to be essential
in the culture of the species in this tribe—as its name suggests. In blood two distinct
and separable substances are present, of which neither alone is able to maintain
growth of *Haemophilus influenzae* (*B. influenzae*), while the two together can do so.
One, the X substance, is associated with the iron-containing fraction of haemo-
globin, and is present also in certain vegetable tissues. It is heat-stable, readily
adsorbed and its mode of action has been correlated with peroxidase activity
(*vide infra*). The other, the V substance, is relatively heat-labile, occurs in numerous
materials as well as blood and has received its name on account of its supposed vitamin-
like nature, though at present proof is lacking for anything more than a general

resemblance to this class of substance. The different needs of the various members of the haemophilic group of organisms for these two factors form a useful basis for their classification, although this has not been adopted universally, *e.g.* by the American Society of Bacteriologists (see Bergey, 1930). Such a classification, suggested by Fildes (1924), can be summarised as follows, and there is need for further work along these lines:

Class	X factor required	Unable to synthesise V factor	Example
A	+	+	*H. influenzae*
B	+	−	*H. canis* (*B. haemoglobinophilus canis*)
C	−	+	Haemolytic *x*-bacilli, *B. parainfluenzae* (Rivers)

The synthesis of these factors (*X* and *V*) by bacteria outside the Haemophileae is discussed in Section V. A full account of the factors is given in the Medical Research Council's *System of Bacteriology* (1929), where mention is also made of a third factor required in artificial cultivation of the influenza bacillus. This is probably of an amino acid nature.

Regarding the Mycobacteriaceae, it is well known to bacteriologists that special factors are required by *Mycobacterium tuberculosis*. The nature of the factors present in potato, which is widely used in the culture of this organism, has been studied by Uyei (*vide infra*). Uyei classified them as (*a*) metabolic and (*b*) reproductive stimulants, the latter alone being capable of stimulating small inocula.

Some of the organisms of the Spirochaetaceae are extremely difficult to cultivate under laboratory conditions. Most of the media which have been suggested include the addition of blood or animal tissue or extracts of these, but so far little light has been thrown on the nature of the factors involved.

A study of the growth requirements of a Streptothrix has been made by Reader (1927 *a*, 1928). It appears from her work that for vigorous growth a special factor is required. It was also observed that the addition of mannitol causes a further stimulation of growth (Reader, 1929).

(b) Fungi.

The earliest studies of the factors required for growth of yeast were made by Pasteur over seventy years ago (Pasteur, 1860), to whom is due the original idea of growth-promoting factors. It was not until 1901, however, that it was first suggested (by Wildiers) that any substance other than sugar and mineral salts was necessary for growth. Wildiers noted that small seedings of yeast barely developed whereas large seedings grew well and subsequently fermented the medium which he used. He assumed that a chemical substance, indispensable for growth, was present in the larger seeding. To this he gave the name "bios." He described some chemical properties of bios and showed that it was organic and present in yeast extract among other sources. During the next six years a fierce controversy was maintained as to whether or not any such substance was essential. From 1907

till 1919, the question received little notice, but was then revived on account of the obvious similarity between the action of bios and that of the "accessory food factors" or vitamins of higher organisms, which had by this time attracted a good deal of attention. The similarity proved to be superficial, and the hopes that had been entertained of using a "bios-test" for assay of vitamin B were soon shattered (see Section IV). During the past ten years the attack on the problem of bios has been renewed, but the effort has not been applied to the essential growth requirements of yeast quite so completely as might be desired. The articles of Tanner (1925), Miller (1930), and Buchanan and Fulmer (1930, p. 545) cover the subject very thoroughly, and from them it is clear that considerable progress has been made and in some cases active substances have been isolated and their constitution determined. It is still doubtful, however, whether the problem of the indispensable substance or substances has been solved.

Among the higher fungi it is only in a few species that growth factors have been shown to be essential. The majority of these organisms can undoubtedly develop normally in the absence of special factors, though numerous cases have been recorded of stimulation of growth by various extracts.

A. Bacteria (Schizomycetes).

Nitrobacteriaceae.

Nitrobacterieae. Mockeridge (1917) found that fractions containing "auximones" stimulate nitrification in cultures of nitrifying organisms. Auximones are plant food accessory substances which are produced in material such as peat by the action of micro-organisms and cause marked stimulation of plant growth (Bottomley, 1914). Beyond a certain maximum of nitrification, inhibition occurred in Mockeridge's experiments which was attributed to accumulation of nitrates, although it is commonly stated in text-books (*e.g.* Jordan, 1931, p. 701) that nitrates have little inhibitory effect. Bottomley (1915) had actually used these organisms in testing for the presence of "auximones" and worked out the distribution and partial fractionation of these substances by means of this test. He admits that the formation of scum, which he used as the criterion of the test, is due to the activity of at least two organisms, so that it is doubtful whether we are here dealing with a clear-cut case of the need of a specific factor by a single organism. There was no evidence that the auximones were needed for initiation of growth. Murray (1923) used autolysed yeast in the culture of nitrifying bacteria. The effect of organic matter in general on nitrification has been the subject of much discussion and is, according to many observers, inhibitory. The question is beyond the scope of this review, inasmuch as the organisms concerned can be grown in purely inorganic media such as were originally used by Winogradsky (1890). The reader is referred to Mockeridge's work (*loc. cit.*) for further details.

It may be mentioned here, in contrast to the above, that in the process of ammonification, Mockeridge (1917) found that no effect either stimulatory or inhibitory was observed as a result of adding "auximone" extracts. She points out

that "it is scarcely to be expected that the activity of these organisms would be affected by substances bearing a close relation to their own products unless these had accumulated in such amounts as to bring about an inhibitory effect." In denitrification processes the extracts were found to be inhibitory.

Azotobacterieae. Bottomley (1915) showed that auximones stimulate growth and nitrogen fixation in the case of *Azotobacter chroococcum*. This was confirmed in the case of pure cultures of this organism, and of *Rhizobium radicicolum* by Mockeridge (1917). Werkman (1927) found that addition of vitamin B concentrate increases the number of organisms during early stages of growth in the case of *A. chroococcum* and *R. leguminosum*. Similar results had been obtained previously by Itano (1923). In the case of this tribe, however, as in the Nitrobacterieae, normal development is possible in the absence of special growth factors.

Coccaceae.

Diplococcus pneumoniae (Pneumococcus). Mueller (1921 a) concluded that the substance essential for growth of the haemolytic streptococci which can be supplied by hydrolysed casein, beef infusion, etc., is probably also essential for *D. pneumoniae*. He also pointed out that batches of meat infusion which failed to give growth of the latter organism could often be improved by adding 0·025–0·05 per cent. of glucose. M'Leod and Wyon (1921) showed that the potency of serum and other materials is diminished by heating, especially in alkali, and that the factor is destroyed by peptic or tryptic digestion. According to these authors, tryptic digests do not enhance growth, while high concentrations may be inhibitory. Webster and Baudisch (cited by Kollath, 1926) suggested that 0·002 mg. per c.c. of "aquosalz" [$Na_2(Fe(CN)_5OH_2) + 6H_2O$, see Hofmann, 1900] could replace blood in the culture of this organism, but Kollath (1926) was unable to reproduce this if solid media were employed in place of liquid. The work of Hosoya and Kuroya (1923 a) is discussed in Section IV. Wright (1929) has observed that the length of the lag phase and the rate of growth of *D. pneumoniae* depend on all the nitrogenous constituents of the medium as well as on the accessory factors. The former substances can be supplied by 1 per cent. peptone, the latter by yeast extract, blood, meat extract or serum. According to this observer, application of heat to organic fluids leads to the production of inhibitory substances.

The rapid dying of *D. pneumoniae* in oxygenated cultures was investigated by Phelon, Duthie and M'Leod (1927) who found the production of hydrogen peroxide to be responsible, in spite of the presence of abundance of catalase.

Streptococci. Kligler (1919) showed that the growth of these organisms was favoured by tissue extracts, *e.g.* beef heart, spleen, liver, etc., and that the length of the lag phase was reduced. Ayers and Mudge (1922) suggested autolysed yeast as a source of growth factors. Whitehead (1926) fractionated caseinogen broth with ethyl alcohol and separated three factors which appeared to be concerned in growth of streptococci. Of these one was inorganic phosphate, in absence of which a lag period of about 24 hours was observed. The other two were thought to be

protein derivatives, one of which underwent marked deterioration on heating (steaming for 12 hours). Other observations are mentioned in Section IV.

Haemolytic streptococci. Davis (1918) observed that these organisms grew well on blood agar or serum agar but not in pure haemoglobin (1 per cent.) solution. M'Leod and Wyon (1921) found that tryptic digests did not improve the growth of haemolytic streptococcus, and that high concentrations of them may inhibit growth. The work of Hosoya and Kuroya (1923 *b*) and that of Wyon (1923) is discussed elsewhere (Section IV).

Mueller (1921 *a*) found that an essential substance for growth was present in hydrolysed casein, beef heart, or beef infusion, and he was able to effect a partial fractionation of it. The essential substance was absorbed by Norit charcoal and precipitated by mercuric sulphate. The filtrate from the Norit treatment, which was inactive, could be reactivated by the addition of peptone, but the latter alone was incapable of supporting growth. Further work (1921 *b*) revealed the presence of two factors which were required to activate the charcoal filtrate. Neither of these was precipitated by phosphotungstic acid (though the results with this reagent were equivocal) but one, named by him "*x*," was precipitated by silver salts: the other, "*y*," was active in concentration of one-twenty-fifth of a milligram per 100 c.c. Later work (1922 *b*) showed that the activity of the *x* fraction was not connected with the pigment nor with the histidine which it contained. Although certain proteins after hydrolysis were active whereas others were not, it was not possible to point to any of the commoner amino acids as the active agent. The amino acid known as methionine or "Mueller's amino acid" ($C_5H_{11}SNO_2$), although isolated from hydrolysed casein and other proteins, was inactive as a growth stimulant (Mueller, 1923).

Freedman and Funk (1922 *a*) showed that beef infusion and extracts of autolysed brewer's yeast contain substances which markedly stimulate the growth of haemolytic streptococci and yeast cells, and are thus closely similar to the bios-like substance of Funk and Dubin (1921)—called by them vitamin D, which must not be confused with the antirachitic vitamin. See also Section IV. The substances are adsorbed by fuller's earth or Norit charcoal from which they can be recovered by treatment with baryta and acetic acid respectively. The growth-stimulating action of protein hydrolysates was thought to be due to the presence of similar factors and not to a constituent part of the protein molecule (1922 *b*). These authors confirmed the work of Mueller (1921 *a*) in showing that a second factor was present in the filtrate from charcoal treatment even after using 10 per cent. of the adsorbent; present indeed to such an extent that the growth-promoting power was unaffected by the charcoal treatment if peptone, which alone permitted no growth, was supplied. The second factor was thought to be associated with blood.

Neisseria gonorrhoeae (Gonococcus). Cole and Lloyd (1917) determined the growth requirements for this organism. They find as essential conditions of growth (1) *p*H 7·6, (2) high concentrations of amino acids and (3) the presence of certain growth hormones. If the *p*H is more alkaline than 7·6, autolysis occurs more readily amongst the cells. Amino acids are essential and the suitability of peptone is due entirely

to the free amino acids which it contains. The peptone is split by enzymes of the bacteria before it is utilised. The growth hormones are probably associated with colloids and may be carried down if these are coagulated. Tissue extracts (meat, spleen, kidney, etc.) induce luxuriant secondary growth but do not affect primary growth and can be filtered without loss of potency. Two growth factors were therefore suggested, A, which is adsorbed easily and occurs in blood corpuscles, and N, non-adsorbed, present in tissue extracts. The latter alone do not increase the number of colonies, but only their size.

The optimum conditions for growth were also studied by Jenkins (1921). In addition to temperature and reaction, the moisture content of the atmosphere and that of the medium were found to be important. Ten per cent. whole blood agar was a satisfactory medium, and later (1922) it was determined that the proportion of serum present need not exceed 1 per cent. Under optimum conditions all strains of the organism developed equally well.

Ainley Walker (1922) showed that addition of alcohol-ether extracts of Marmite (a commercial yeast concentrate) or egg-yolk improved the growth of *N. gonorrhoeae* and that other less delicate organisms were capable of producing substances stimulatory to this organism. Morini (1920) had previously used extract of yeast in cultivation of this organism.

The later work of Jenkins (1924), which seems to have been rather overlooked, showed that the best test of the value of a medium for growth of *N. gonorrhoeae* lay in the study of the first few subcultures. Many media allowed good growth after a few subcultures, presumably on account of acclimatisation of the organism. Moreover, inadequate media might give good growth in primary culture, on account of the pus which accompanied the inoculated organism. The specific substances in serum which were necessary for growth, according to the criteria of Jenkins, were found to be destroyed by heat in two clear stages. Two factors were therefore thought to be responsible: $G\,1$, which was destroyed at 65° C., and was found only in blood and its derivatives, and not in yeast or muscle; and $G\,2$, which was destroyed above 100° C. and was present in blood (serum more than corpuscles) and muscle. From the latter $G\,2$ requires extraction by digestion with certain acids. These substances were compared with those which had been described by Thjötta and Avery (1921 *a* and *b*) for *H. influenzae*. $G\,1$ is obviously a different substance from X of these authors, and $G\,2$ only resembles V in so far as it is destroyed at temperatures above 100° C. The substances described by Jenkins cannot be correlated with the A and N substances of Cole and Lloyd.

Gordon (1926) showed that incorporation of 0·1 per cent. of taurine renders the blood-agar medium more suitable for growth. The medium he used had been sterilised by heating at 70° C., but there is no reason to assume that taurine is identical with Jenkins's $G\,1$. Gordon suggested that on account of its power of lowering surface tension taurine would be concentrated at the surface of the organisms (Rideal, 1923) and hence be available as a source of nutrition. This suggestion as to the mode of action does not seem adequate in cases of stimulation of growth by factors which are specific, as in the case of *N. gonorrhoeae*, for, as

was shown in Section II, many types of substance can cause lowering of surface tension. It is doubtful, too, whether taurine would be so concentrated, and the possibility of oxidation-reduction effects may be mentioned.

Opposed to the observations of Jenkins, are those of M'Leod, Wheatley and Phelon (1927), who showed that whole blood heated between 55 and 100° C. is better than unheated. They included meat extract in their medium; thus the G 1 factor of Jenkins would be absent. Peptone was found to have an inhibitory effect unless blood or a colloid, such as may be supplied by ascitic fluid, was present. The colloid was thought to have a protective effect (cf. M'Leod and Wyon, 1921). The authors were unable to separate from the meat extract the organic substance which exerted a favourable effect. Their paper contains a valuable bibliography.

Tempé (1926) showed that an extract of *Mucor mucedo* contains a stimulatory factor for *N. gonorrhoeae*, and suggested a medium in which the extract was combined with haemolysed blood. The composition of this medium is undoubtedly complex, having as its basis a boiled water extract of liver. The factor of *Mucor mucedo* was found to be thermostable (resisting 120° C.) and to pass through a Chamberland filter. Thus it is apparently unlike any of the factors mentioned above resembling only the N factor of Cole and Lloyd. Its action appears to be similar to that of mannitol in the growth of *Streptothrix corallinus* (Reader, 1929).

Rapid death of *N. gonorrhoeae* in oxygenated cultures was shown by Phelon, Duthie and M'Leod (1927) to be due to the operation of two factors as in the case of *N. intracellularis* (*q.v.*).

Neisseria intracellularis (Meningococcus). Gordon, Hine and Flack (1916) showed that the growth factor for this organism which occurs in blood is not destroyed by heating and that it can be replaced by various proteins, for example, serum albumin or fibrinogen. They evolved a medium composed of trypsinised broth and pea-flour extract which was suitable for primary culture from cerebro-spinal fluid and for growing the organism in bulk. Addition of serum to this medium caused an increase in the size of colonies but not in their number. Legroux (1920) described a medium ıor this organism composed only of diluted serum.

The work of Kligler (1919) and Hosoya and Kuroya (1923 *a*) is referred to in Section IV. In connection with the relation between vitamins and the growth factors of this organism, it may be mentioned here that Gordon, Hine and Flack classified the pea-flour factor provisionally as a vitamin, but pointed out that extract of wheat germ, which is typically vitamin-containing, tended to prolong the vitality of the organism in culture rather than promote growth. Similarly Eberson (1919, 1920) described a yeast agar on which the organism would survive for as long as five months, with little alteration, if any, in its antigenic properties.

Lloyd (1916) showed that the accessory factors present in blood, milk, etc., which are considered essential for the primary culture of the organism *in vitro* are moderately heat-stable. This was confirmed by M'Leod and Wyon (1921). She also showed that these factors are soluble in water and 80 per cent. alcohol, and adsorbed by filter paper. According to her work, the potency of material such as

blood is not due to protein. The amount of growth factors required was found to vary inversely as the amino acid content of the medium, and it was suggested that their mode of action is to increase proteolysis. M'Leod and Wyon (1921) found that the active substance was not extracted by 95 per cent. alcohol, and was destroyed by tryptic or peptic digestion. These authors state that tryptic digests do not enhance the growth of *N. intracellularis*. Wyon (1923) states that on addition to peptone-meat-extract agar charcoal has a similar effect to blood, and acts by removing inhibitory substances.

In oxygenated cultures *N. intracellularis* rapidly dies. The factors concerned have been investigated by Phelon, Duthie and M'Leod (1927) and were found to be: (1) the development of a markedly alkaline reaction, (2) the production of a second factor independent of alkalinity, the nature of which remains obscure.

Staphylococci. Goy (1925) noted that the growth of a staphylococcus was improved by an extract of *Amylomucor β*. Taking into account the amino acid content of the medium, Wyon (1923) showed that a vitamin B concentrate slightly improved the growth of *S. albus* and *S. aureus*, though the work of Gordon and M'Leod (1926) indicates that the growth of *S. aureus* is not affected by the presence of amino acids. Other work is discussed in Section IV.

Bacteriaceae.

Cellulomonadeae. Sanborn (1926 a) noted stimulation of the growth and cellulose decomposition of *Cellulomonas folia* in the presence of various extracts (extracts of germinated and ungerminated seeds, yeast, etc.). Changes in the hydrogen-ion concentration were studied as the criterion of the physiological efficiency of the organism and later (1927 b, 1928) a special medium was described in which a suitable indicator was incorporated. Active extracts could also be prepared from other bacteria and various plants. From decomposing plant material the essential substances pass into the soil, as shown by Sanborn (1927 a). Although in Sanborn's work the stimulation of growth was in no case great, the stimulating effect was more significant than is apparent at first, since in the control cultures the organism died out more or less rapidly. Thus in the medium used by Sanborn the stimulating substance was thought to be essential, though it must be pointed out that various workers have found that cellulose decomposition is largely controlled by the amount of available nitrogen present (see Werkman, 1927).

Haemophileae. The subject of the accessory growth factors for this group has been fully reviewed in the Medical Research Council's *System of Bacteriology* (1929). The author will therefore confine himself to the following additions to, and comments on, this article, which will be referred to as the "M.R.C. account." The subject will be dealt with under the same headings.

Historical. In the M.R.C. account, as in many other references to the subject, the work of Davis (1917) is left in the background, while the later confirmation of it by Thjötta and Avery (1921 a) is brought to the fore. In fact, many of the results put forward by the latter workers had been previously established by Davis.

He had pointed out that haemoglobin is essential for growth; that it is active in high dilution (from 1 in 100,000 to 1 in 180,000); that it is inadequate alone, and that it does not entirely lose its growth-promoting property through heating at the boiling-point or even at higher temperatures. He showed that haemoglobin alone will allow of some growth, but when it is associated with certain bacteria or plant or animal tissues, an optimum growth is obtained although the latter alone will not support growth. He compared the two factors to the "fat-soluble A" and "water-soluble B" of McCollum and Davis (1915), but did not push the comparison further than to say that they may be analogous to those two vitamins.

The M.R.C. account of Fildes' work (1921) in which the separate nature of the two factors was further confirmed does not make it clear that the precipitated haematin fraction was actually haemin (haematin hydrochloride). Haemin alone failed to support growth, but on addition of supernatant fluid, which contained only traces of pigment, copious growth resulted.

Thjötta and Avery (1921 *b*) are cited as being the first to prove that growth is not absolutely dependent on blood pigment. As Thjötta himself points out (1921), Cantani (1897, 1901) had claimed more than twenty years previously to have obtained growth by the use of spermatic fluid and other materials, *e.g.* egg-white, or yolk, and (1902) by the addition of other bacteria. Although the evidence refuting this (Ghon and Preyss, 1902, 1904; Luerssen, 1904, and others) seems rather overwhelming, Cantani mentions other authors who have confirmed his observations. In this connection Fildes (1921) states that Cantani's mistake was that of using the spectroscope as a test for blood pigment, but he himself does not suggest any other than the guaiac test, which is not specific, and is thus no more reliable although it is undoubtedly more sensitive. Cantani's work is, moreover, confirmed in part by the observation of Thjötta (1921) that "*H. influenzae* will grow profusely in plain broth containing extracts of mucoid bacilli or *B. proteus*." As the work of Rivers and Poole (1921) is specially mentioned in the M.R.C. account, it should be recorded that Davis (1921 *b*) had independently shown the same result.

Experime. tal. In discussing the importance of carefully controlled experimental technique, the M.R.C. account states "one must make certain that the inoculum is not too small in proportion to the bulk of medium inoculated; it is well known that many bacterial species inoculated in minute amount in a medium quite capable of maintaining their growth may fail to develop." Such a statement is misleading, as an adequate medium for a given bacterial species should be capable of permitting development of even the smallest inoculum. The question is further discussed in this section (part B). It is doubtful, too, whether "it is probable, in fact, that all bacteria require some substance—growth factor or enzyme—to be present in sufficient concentration before they can start to metabolise their food material" as many species have been cultured successfully on synthetic media, which must be assumed to be free from growth factors at least until the contrary is proved.

The V factor. It does not appear to be true to state that "it (the *V* factor) is capable of passing through filters without serious loss, *i.e.* it is not readily adsorbed," for it was the loss, recorded in the earlier literature, of the factor during

filtration that led Thjötta and Avery (1921 *a*) to test adsorption on charcoal, which they found to be almost complete at *p*H 5·4, especially on warming.

Comparison with vitamin C. In its origin, the *V* factor certainly resembles vitamin C, but not so closely as the M.R.C. account suggests. The *V* factor was found in bananas (Thjötta and Avery, 1921 *b*), meat infusion (Rivers and Poole, 1921), legumes and yeast (Thjötta and Avery, 1921 *a*; Fildes, 1923), carrots (Kent, 1923) and various bacteria (Kollath and Leichtentritt, 1925, 1926). These are all poor sources of vitamin C (M.R.C. Committee on Vitamins, 1924).

Mode of action. "The suggestion of Lloyd (1916) that the accessory substances may act as a catalyst accelerating the splitting of nitrogenous substances" was made when she was studying the accessory substances required by *Neisseria intracellularis*, and there is no suggestion in her work that such a mode of action should be applied to the case of any other organisms. *Haemophilus influenzae* was only mentioned in the last paragraph of her article and then in a different connection. The alternative mode of action suggested in the M.R.C. account, that the *V* factor is essentially an organic peroxide, is hard to reconcile with its susceptibility to oxidation coupled with its stability at ordinary temperatures.

The X factor. Neisser (1903) succeeded in cultivating influenza bacilli in symbiosis with *Corynebacterium xerosis* which may be interpreted as showing that the latter organism can produce the *X* factor, "symbiosis in this instance meaning that the colonies were actually mixtures of the two bacteria." Neisser, however, points out that the influenza bacillus usually grew at the edges of the *C. xerosis* colonies, and Davis (1921 *b*) has confirmed Neisser's work but was emphatic in stating that there was no true symbiosis as *C. xerosis* does not benefit from its association. Moreover, he states that growth of *H. influenzae* is not so good if the "symbiotic" organism is mixed thoroughly with it (see also Rivers and Poole, 1921).

The factor is said to be "associated with a peroxidase action" and the statement is made that the coincidence of peroxidase activity with *X*-factor activity is perfect "with some explainable exceptions." It is stated earlier (in the M.R.C. account) that "autoclaved vegetable tissue generally is devoid of peroxidase activity though still sufficiently active as *X* factor." This exception is explained very inadequately by the assumption that influenzal growth is a more delicate indicator than the peroxidase test (benzidine or guaiacum). It must be remembered that the *X* factor is thermostable, whereas thermolability is characteristic of vegetable peroxidases in general whether or not the benzidine reaction is used to detect their presence (see Stephenson, 1930, p. 61). Moreover Davis (1921 *a*) pointed out that many iron compounds (and other substances) give a positive peroxidase reaction but do not contain *X* factor (Davis's factor 1). Thjötta and Avery (1921 *b*) found that banana contains both *X* and *V* factors, but gives no benzidine reaction. Again, Kent (1923) states that many vegetable tissues which do not give the test support growth. It seems therefore that the correlation of peroxidase activity with the mode of action of the *X* factor (first suggested by Olsen (1920), not by Fildes) cannot be accepted as entirely correct. It is of interest that Lwoff (1931) suggests

that the substance in blood essential for the growth of trypanosomes is a per-oxidase, identical with, or very similar to, the X factor.

The close association of the factor with iron has been repeatedly confirmed, although it is denied by Tokugana (1920). In the M.R.C. account it is suggested that the phenomena of iron starvation in plant nutrition may partly depend on the absence of some such factor as the X factor, and that no substance in animal nutrition is comparable. It seems to the author reasonable to suggest that the phenomena of certain types of anaemia in man are due to lack of a similar factor. In these the therapeutic value of liver treatment has now been established while simple iron compounds are of little avail (Witts and others, 1931; Vaughan and others, 1931). This suggestion is supported by the observation of Sergent (1928, pp. 70, 71) that addition of bile to ordinary media renders them capable of sup-porting growth of *H. influenzae* (cf. the effect of liver extract on etiolation of plants observed by Raber (1931)).

Sergent (1928, p. 85) made a study of the stimulation of *Haemophilus ducreyii* by the body fluids in various pathological conditions, and found no apparent modification of the factors in blood from a variety of cases.

Bacterieae. The organisms in the colon-typhoid group, as is well known, can develop in media of simple composition, and there is no work on specific growth factors which needs discussion here. If, however, the reader is interested in sub-stances which increase the rate of early development of the organisms (that is, during the first six hours) he should consult the article by Knorr (1925 *a*).

Mycobacteriaceae.

Mycobacterium tuberculosis. Uyei (1930) studying the growth of tubercle bacilli (human) *in vitro* showed that addition of potato to a modified Long's medium rendered it suitable for the growth of far smaller inoculations than could be grown in Long's medium alone. This property of potato was not destroyed by autoclaving, nor removed by extraction with organic solvents (*e.g.* acetone, alcohol, or ether). Investigation of the effects of known constituents of the potato showed that neither the protein, glycogen nor the ash were involved. The active constituents were classed as (*a*) metabolic stimulants, namely, inosite, maltose and glucose, which only act with large seedings of the organism, and (*b*) reproductive stimulants, namely soluble starch and dextrin, which stimulate the growth of both large and small seedings.

The necessity of carbon dioxide for the cultivation of *M. tuberculosis* has been emphasised by Wherry and Ervin (1918) and Rockwell and Highberger (1926).

Spirochaetaceae.

Twort (1921) showed that primary cultures only survived in the presence of the gland tissue which was used as the source of these organisms, or in standard egg medium to which had been added an emulsion of *Mycobacterium phlaei*. In older cultures on the latter medium, however, the typical spirochaete form became

progressively less numerous, its place being taken by involution forms. No growth resulted on ordinary media even with addition of 10 per cent. blood, though, in the latter case, preliminary incubation of the medium seemed to improve it, and some successful cultures were obtained. Not all spirochaetes could be successfully cultivated on the special media (*e.g.* those from the stomach of normal cats and dogs) and some could be acclimatised to grow in the egg medium without the addition of *M. phlaei*. The growth requirements of *Leptospira icterohaemorrhagica* (*S. ictero-haemorrhagiae*) have been studied by Noguchi (1917) who found that the use of a piece of fresh tissue, which is so advantageous in growing other spirochaetes, might be seriously detrimental to this organism. Stefanopoulo (1921) prepared an active extract from red blood corpuscles.

Chlamydobacteriaceae.

Streptothrix corallinus. Reader (1927 *a*) has made a detailed study of the growth requirements of this organism. She used the same organism to study the effect of changes in surface tension (1927 *b*). This is referred to elsewhere (Section II). Later, Reader (1928) reported that for vigorous growth a special factor is required. This could be obtained from fresh beef, wheat germ and preparations of yeast. The factor was found to be organic, water soluble, dialysable, and in purer preparation, stable to alkali. Its relation to antineuritic vitamin will be discussed in Section IV.

B. FUNGI.

Yeasts.

The question of growth factors of these organisms resolves itself into a discussion of what has now become generally known as the "Bios problem." The published work up till 1924 has been fully reviewed by Tanner (1925). To this comprehensive article the author suggests the following corrections, amendments and additions. It will be necessary first to recapitulate briefly the original observations of Wildiers which led him to assume the necessity of bios for yeast growth, though this has already been carefully done by Tanner. Part of the material presented here is due to appear elsewhere (Peskett, 1928 *a*).

Wildiers (1901) found that with small inoculations *practically* no growth or fermentation occurred, whereas with large seedings normal growth and fermentation took place. In the latter case it was assumed that "bios," a chemical substance which was considered to be indispensable for growth (and fermentation), was present. On the basis of these results Wildiers sought to explain the older controversy between Pasteur (1860) and Liebig (1871) by assuming that Pasteur had used a larger inoculum than Liebig. It will be remembered that Liebig repeatedly failed to confirm Pasteur's observations that yeast grows on a medium composed of simple salts and sugar. Wildiers' conclusions were met at first by a great deal of opposition (see Tanner, 1925), largely because it was erroneously assumed that he had claimed that with small inoculations *absolutely* no growth or fermentation occurred. Such an assumption was not justified, as several authors

have realised (see Tanner, 1925; Miller, 1930). In fact Wildiers' findings (*vide supra*) whether or not they were clearly stated by him, are undoubtedly supported by the following experimental evidence. In the first place there is the valuable work of Kossowicz, one of whose papers seems to have been overlooked. As a diminution in power of multiplication had been noted with diminution in size of inoculum (Kossowicz, 1903 *a*), further experiments were carried out with single cells as seedings (1903 *b*). After one year slight development of the yeast was visible in only one out of the twenty flasks of the salt-sugar solution that had been inoculated with single cells, whereas control flasks which had been seeded with large numbers showed rich development. Kossowicz concluded that: (1) with very small inoculations there was no multiplication nor fermentation, (2) with greater inoculations (over 100 cells) slight multiplication occurred but no visible fermentation, (3) with large seedings (1,000,000 cells) both multiplication and fermentation took place. The papers of Kossowicz (1903 *a*; 1906) contain a valuable discussion of the work of other authors, especially those of the last century, and include several observations not mentioned in Tanner's review. Secondly Peskett (1928 *a*) showed that the degree to which a seeding of yeast multiplied in a medium composed of mineral salts and purified cane sugar was constant (10–12 times in the case of the species used). In other words, the crop obtained was directly proportional to the size of the seeding. This applied equally to single cells and large inocula. It is probably true that similar growth of the small inoculations actually occurred in Wildiers' experiments, but that his technique only allowed him to observe "gross changes such as are visible to the naked eye" as remarked by Kossowicz. Using a turbidity method of estimating yeast growth, Peskett (1927 *a*) has found that the minimum concentration of his yeast which will show visible turbidity is approximately 500,000 cells per c.c. Ten times this concentration shows, on the other hand, very dense turbidity; thus a culture containing say 100,000 cells per c.c. will not show turbidity (*i.e.* "growth" in Wildiers' opinion), while a culture containing more than 500,000 cells per c.c. will show quite marked "growth."

So far the author has dealt only with the growth of yeast on "synthetic" media. Summarising, it may be said that under these conditions growth is proportional to the size of seeding, but is, at any rate in the case of some yeast species, extremely slow. Consequently with very small seedings, no visible crop will result, whilst with large, a fair visible crop will be obtained.

Turning now to the growth of yeast in the presence of bios or other unknown organic material, several points need consideration. The crystalline bios of Eddy, Kerr and Williams (1924) is a substance isolated by them from yeast extract, having the formula $C_5H_{11}NO_3$, and melting at 223°. According to their results this substance exhibits marked activity as a stimulant of yeast growth. On p. 460 of his article, Tanner states that Peskett found "that Eddy's crystalline 'bios' did not stimulate the growth of yeast. In fact in the mount to which it was added there was less growth than in the mounts which did not contain it." [In the experiments referred to (Peskett, 1924) the growth of single yeast cells had been studied in hanging-drop cultures, called by Tanner "mounts."] These experiments

were not, however, comparable, as in two of them the mounts which contained the hanging-drop cultures without bios had been incubated longer than those with bios, and in the third experiment commercial cane sugar was used which evidently contained some growth stimulant. Peskett did not state that "Eddy's bios does not stimulate the growth of yeast" although these experiments have, on other occasions (Miller, 1930; Eddy, private communication) been interpreted as showing such an effect. See, moreover, later work by Peskett (1928 *b*).

In attempting to summarise the conflicting data and opinions that have been published on the bios problem, Tanner rightly stresses the importance of keeping clear definitions in mind in considering growth factors (see Fulmer and Nelson, 1922; Drummond, 1924). The discussion in Section IV of the present article shows that bios is in all probability not one of the known vitamins, but the possibility of it bearing to yeast growth the same relation as vitamins do to animal growth cannot be excluded. Much of the complexity that has arisen can be traced back to a careless interpretation of the original definition of bios as given by its discoverer "*Nouvelle substance indispensable au développement de la levure*" (Wildiers, 1901). "Indispensable" is surely sufficiently unequivocal! How shall *développement* be defined? Speaking generally, the bacteriologist does not consider that he has satisfied the nutritional needs of an organism until he can take a small loopful of it, inoculate into a comparatively large volume of medium, and then secure such an abundant growth that it can be seen by the naked eye, or by means of a low-power lens. The author has found that the volume contained by an ordinary loop is about 2 c.mm. ($\pm$ at least 50 per cent.). Assuming the yeast cell to be a sphere of diameter $10\,\mu$, a loop filled with yeast would not contain more than four million cells. This amount, which would be considered as a heavy inoculation by the average bacteriologist, seeded into 100 c.c. of medium (which was the volume Wildiers used), would give an initial concentration of 40,000 cells per c.c. Yet in most of the work that has been directed towards determining the food requirements of yeast, the inoculum has been at least 250,000 cells per c.c., *e.g.* Fulmer, Nelson and Sherwood (1921), Clark (1922), Fulmer, Nelson and White (1923), Lucas (1924 *a, b*), Eddy, Kerr and Williams (1924), Devereux and Tanner (1927). The criteria of Wildiers were presumably those which the bacteriologist usually applies to bacterial growth. Let them therefore be applied in defining *développement* of yeast.

Miller (1930), pointed out that Wildiers did not say "A few cells will not grow where many will," but "if the cells have all they need 10,000 should be but few hours behind 50,000". The author suggests that the latter remark can be applied to single cells as follows. If a yeast cell has all it requires, it (that is to say, a single cell or at the most a few cells) should develop to a crop amounting to at least 5,000,000 to 10,000,000 cells per c.c. of medium given suitable conditions of temperature, etc., and sufficient time. With such considerations in mind, bios can still be defined as the substance or substances indispensable for the growth of yeast and this definition should be strictly observed.

The more recent work on bios has been reviewed briefly by Miller (1930) and

Buchanan and Fulmer (1930, p. 545). Miller points out rather dogmatically that the following conditions are essential in testing for bios: (1) the inoculum must be from a culture in the logarithmic stage of growth, and (2) the amount of bios must be such that logarithmic reproduction passes sharply into quiescence. The slope of the logarithmic curve and the crop obtained when logarithmic reproduction ceases are the important matters, the former being characteristic for a given preparation and the latter measuring the amount of active substance present. A yeast should be chosen which buds slowly without bios and quickly with it. Miller seems to pay little attention to the size of the seeding, although in the experience of Peskett (1928 b) a large seeding may grow well under conditions which will not allow more than a very meagre proliferation of a small inoculum. The substances which are essential for the good growth of a small inoculum are of a true bios character. In many cases, the effects produced by substances affecting logarithmic reproduction may be due to bios, but often they are due to growth stimulants and not to bios as defined above, especially if the inoculum is large. The following analogous examples deserve mention. Mannitol has been shown to have such a stimulatory effect in the growth of *S. corallinus* by Reader (1929). "Bios I" described by Lucas (1924 b) was shown by Eastcott (1928) to be inositol, and from her experimental results it is evident that its action on yeast is that of a growth stimulant, not that of a bios. A similar action of inositol was observed by Uyei (1930) in the growth of *M. tuberculosis*. See also the discussion by Darby (1930).

Putting it another way, bios must be considered as an important factor in the early stages of budding. Before labelling any substance as "bios" it must be shown to be capable of inducing the logarithmic phase of growth (Buchanan, 1918; Buchanan and Fulmer, 1928, chap. 2) in addition to any effect it may have on the rate or duration of that phase.

The method of bios testing devised by Peskett (1928 b) consisted in determining the minimum concentration of the test material which would allow a small seeding to attain the same crop as a large seeding. Neither volume of medium nor time of growth was found to affect the result beyond a certain minimal limit which was necessary in the case of time. Although the rocker-tube method (Clark, 1922) was not used, the results obtained fully confirmed Clark's results. The actual quantity of the ultimate crop produced was of no import in this test. The principal advantage in Peskett's method, compared with Miller's, lies in the fact that it measures the ability of the test material to allow growth of a small seeding. The ideal appears to be a method of testing in which the principles of both Miller's and Peskett's methods are included.

Whilst dealing with methods of testing it must be pointed out that nothing has hindered the solution of the bios problem more than the use of fermentation measurements as a means of assaying bios activity. The two activities—growth and fermentation—frequently run parallel, but by no means invariably, as has been clearly shown by Euler and Petterson (1921) [cf. later work of Philipson (1930)]. There is now little difficulty in separating the two activities. Agencies

are known which inhibit growth without seriously affecting fermentation, *e.g.* Röntgen rays (Wels and Osann, 1925), phenol (Euler and Sandberg, 1925). Acetone-yeast or other non-living preparations can be used to study fermentation *per se* (Harden, 1923) whilst Cayrol and Genevois (1931) have recently shown that the presence of 14 mg. per litre of bromoacetic acid causes a specific inhibition of fermentation without arresting other activities. Nevertheless workers continue to use fermentation measurements for the assay of bios activity, *e.g.* Suzuki and co-workers (1930).

The following recent observations need mention. The suggestion of Darby (1930) that the pH of Wildiers' medium (7·2–7·4) is too far removed from that of the optimum for yeast (4·4) does not explain the inability of yeast to grow rapidly in media (pH 4·5 approx.) similar to Pasteur's which were used by Peskett (*vide supra*). When bios was added to such media excellent growth was obtained. Narayanan (1930) reported the result of testing the bios activity of a variety of substances all of which were inactive, including the α and β bioses of Eddy, Kerr and Williams (1924) and inositol; the inactivity of the latter has been confirmed by Peskett (unpublished data). It is noteworthy that Narayanan used comparatively small inocula (2500 cells per c.c.). He described a method by which he obtained preparations active in concentrations as low as 0·01 mg. per c.c. These appeared to consist largely of comparatively simple nitrogenous substances. His paper contains an interesting table in which the properties of his preparations are compared with those of some of the earlier workers. No evidence was obtained in support of the complex nature of bios which has been suggested by several observers. Similarly, Peskett and O'Brien (1930) could obtain no evidence in support of the multiple nature of bios. They reported a method of fractionation resulting in a preparation which was active in doses of 0·005 mg. per c.c. for even smaller inoculations than Narayanan used. Williams and Bradway (1931) reported on the growth of cultures of the same yeast that Wildiers studied, and compared its growth needs with those of the Gebrüde Mayer yeast which they used. They found that the growth stimulant of these two yeasts differed from those of others in that its activity was not appreciably altered by treatment with fuller's earth, nor could conclusive evidence of its multiple nature be obtained. They compared Gebrüde Mayer yeast with those used by Narayanan and Peskett and O'Brien, though they were erroneous in stating that the latter observers used baker's yeast. The general conclusion suggested by this work is that those workers who have reported multiplicity among the growth factors required for yeast have not been studying the bios problem as originated by Wildiers. Later, however, Williams and Truesdail (1931) reported that they had fractionated Wildiers' bios itself into two factors by electrolytic means.

Elvehjem (1931) studied the rôle of iron and copper in the growth of yeast. His work suggests that, although both iron and copper are essential to, and may cause marked acceleration of, growth, it must not be concluded that herein lies the solution of the bios problem. The majority if not all of the synthetic media that have been used probably contain an adequate supply of these elements. Elvehjem

considers that part of the action of bios on growth may be dependent on changes which make the iron more available under conditions of high pH. Greaves, Zobell and Greaves (1928) had shown previously that iodine was essential to yeast growth but concluded that "it cannot be stated that iodine meets fully the requirements of bios." Stimulation by iodine is confirmed by Kooijmans (1930), but this observer notes that normal development can occur in the presence of yeast extract though the amount of iodine present is far less than that which Greaves and his colleagues state to be necessary.

In summarising, Tanner (1925) divided those who have made investigations in the bios problem into four groups according to their opinions:

(1) Those who affirm the existence or need of bios.
(2) Those who deny it.
(3) Those who state that yeast can grow without bios but admit that growth is improved by addition of it.
(4) Those who have isolated substances which have bios properties.

If Wildiers' definition be adhered to, these groups can be at once reduced to two:

(1) Those who affirm the existence of bios.
(2) Those who deny it.

The question naturally arises, is it possible to reconcile two schools of thought which appear to contradict each other so completely? Seventy years ago, in the days of the Liebig-Pasteur controversy it would have been impossible. Ten years ago it would have been barely possible, as the two views were held each practically to the exclusion of the other. To-day there is evidence that some races of yeast can develop without bios whereas others cannot (Lucas, 1924 *b*; Tanner, Devereux and Higgins, 1926; Devereux and Tanner, 1927; Copping, 1929). Thus the contradiction, which was probably based on the false assumption that all yeasts act alike, ceases to exist.

The bios problem obviously exists in connection with those yeasts which cannot develop in a synthetic medium (in which the chemical composition of all the constituents is known). As regards those races which are said to develop without bios, the author knows of no recorded experiment in which a culture has been started from a small inoculation (of less than one thousand cells) and maintained through repeated subculture on a synthetic medium, using in each case a very small inoculum; so the bios problem may apply in the case of these races too.

It may be added that the very striking "bios effect" in which a large seeding develops out of all proportion to a small, is only to be observed in the presence of bios, and does not appear in truly "synthetic" media.

Higher Fungi.

There is little doubt that the majority of organisms of this class can develop on media of known composition. This has been well established in the case of *Aspergillus niger*, for example, by Raulin (1870), Currie (1917), Raistrick and Clark (1919), Goy (1921, 1922 *a*, *b*). At the same time stimulation of growth has been noted in

the case of this organism by addition of various extracts, *e.g.* of yeast even after heating to 130° C. (Lumière, 1921), of the mycelium or the spores of the organism itself (Currie, 1917) or of other organisms (Linossier, 1920; Goy, 1921; 1922 *a, b*). The growth factors of such extracts are obviously not essential. On the other hand, in the case of *Sclerotinia cinerea*, Willaman (1918, 1920) found that it cannot develop on a synthetic medium but that yeast extract can furnish the essential material.

For a full review of the requirements of *Aspergillus niger* in relation to traces of metals the reader is referred to the paper by Roberg (1931).

IV. RELATION OF FACTORS TO VITAMINS.

SUMMARY.

Bacteriological media being in the main aqueous, it is evident that it is chiefly in the class of water-soluble vitamins that one must look for possible relationships between bacterial growth factors and vitamins. In this class it will be found that the B group has attracted by far the greatest attention. There is some evidence that vitamin C may influence growth, but it is as yet very meagre (*vide infra*).

There is no doubt that the majority of lower organisms respond with improved growth to the addition of extracts which contain vitamin B. This was first noted at least fifteen years ago by Lloyd (1916), and it is not surprising that in several instances it was assumed that the active material involved was the vitamin itself. The fact that yeast behaved as the majority of other lower organisms in this respect; that an indispensable substance, bios, was thought to be required for its growth and that it was recognised as a very rich source of the vitamin, led to the appearance of a "yeast test" for vitamin B in which stimulation of growth of yeast (and even of its fermentative activities) was used as the criterion for assay of vitamin. The subject is discussed briefly in the section which follows, and a full account of it is given by Tanner (1925). On the other hand, as early as 1918 it was noted that the assumed identity of the growth factors of lower organisms with vitamin B was open to doubt, and since that date considerable evidence has accumulated to disprove the identity. The yeast test has had to be discarded as unreliable though it may be remarked that a "Streptothrix test" (*vide infra*, p. 31) has been usefully applied by Peters, Kinnersley, Orr-Ewing and Reader (1928) in testing preparations during fractionation of the antineuritic vitamin (B_1).

Only in a few cases has it been shown that the addition of vitamin extracts was essential to growth so that their action on lower organisms cannot be identified with that which occurs in higher organisms. Even in these few instances in which vitamin extracts have been shown to provide an indispensable growth factor there is no definite evidence that it is the vitamin which is the active agent. The problem of relationship between the growth factors and vitamins must remain open until the various members of the B group of vitamins or the factors in question are obtained in pure state. Some progress has been made in this direction, resulting in subdivision of vitamin B into various fractions (see *Nature* (1931), **127**, 95, 131

and 204, and in the partial purification of some of the growth factors, but it appears that none of the factors for lower organisms is identical with any of the vitamin B fractions as far as can be judged from the data available at present.

A. FAT-SOLUBLE VITAMINS.

Uyei (1927 *a*) found that a cod-liver oil concentrate ("Oscodal" of Funk and Dubin, 1923) known to be rich in vitamins A and D exerted only an indifferent effect on the growth of tubercle bacilli. Sergent (1928) reported experiments on a race of *N. asteroides* (Eppinger) which had lost its acid-fast characteristic. It regained this property when repeatedly subcultured on media containing olive oil, but from his report it appears that cod-liver oil did not have a similar effect (cf. the results of Miller (1916) in the case of *M. tuberculosis*). There appears therefore no justification for his conclusion that oils containing vitamin A partially restored "acid-fastness" to the organism which had lost this characteristic since olive oil contains no vitamin A or D. At present there seems to be no evidence that fat-soluble vitamins are among the factors affecting growth of lower organisms.

B. WATER-SOLUBLE VITAMINS.

Gordon, Hine and Flack (1916), studying the growth needs of the meningococcus, were among the earliest to classify as vitamins the essential growth substances in contrast to the substances in wheat-germ extract which prolong the vitality of the organism. Lloyd (1916) similarly classified the factors of blood, serum, milk, etc., which promoted growth of this organism.

Davis (1918) was one of the first to point out that vitamins are of doubtful importance in the growth of those organisms which require special factors. He studied the growth of *B. coli*, *B. typhosus*, *B. diphtheriae*, *Streptococcus haemolyticus*, *Staphylococcus aureus*, *Blastomycetes*, *Sporotrichum Schenkii*, *Streptothrix*, *B. pyocyaneus* and *B. prodigiosus*. Some of these organisms had already been grouped as obligatory haemophils (Davis, 1915), but some are now known to be capable of growth in synthetic media. All the species studied, except *B. diphtheriae* and the streptococci, grew moderately in pure haemoglobin (1 per cent. solution) and all grew well on blood- or serum-agar. Ordinary meat extract agar, known to be inadequate as a medium for some species, was not improved by addition of extracts of rice flour (polished or unpolished) or wheat bran, but all the species which Davis tested grew on extracts of polished or unpolished rice flour. Moderate growth was also obtained on extracts of white and whole wheat flour. Although there were, in some cases, quite marked differences in the growth with these various grain products, these differences could not be correlated with the known vitamin content.

Kligler (1919) studied the growth-favouring action of various tissue extracts on the meningococcus, pneumococcus, a streptococcus, *B. diphtheriae*, *B. pertussis* and *B. influenzae*. As the factors concerned were found to be water soluble, heat labile, of a non-protein nature, Kligler placed them in the class of vitamins. He pointed

out that their action was not to provide food material since no growth occurred on diluted extracts (five times) such as he expected if the original material were rich in nutritive substances. This result might equally well be interpreted as showing that the original material was deficient in growth factors.

Since it was at this time that fresh interest was awakened in bios [the factor required for yeast growth (see Section III)], on account of its possible relationship to vitamin B, it will simplify the discussion if this question is considered in full before returning to the bacterial factors. Kurono (1915) had shown that vitamin B (oryzanin) extracts stimulate yeast growth, but it was not till Williams (1919, 1920) published his method of testing for vitamin B by the measurement of yeast growth that serious work was undertaken on these lines. The subject has been thoroughly covered by Tanner (1925) and throughout his article, except in the first twenty pages, numerous references to it will be found. There is neither the space nor the necessity to recapitulate this work here beyond giving the following summary:

The hypothesis underlying Williams's method was that bios and the antiberi-beri (or antineuritic) vitamin are identical. The experimental technique used underwent various modifications in the hands of several investigators, but all methods assumed the identity of the two factors. In some cases the assumption was extended to include fermentative activity of the yeast as being identical with bios activity. Thus the apparent simplification of vitamin testing which Williams introduced resulted in considerable complication, as the methods devised were often less comparable than the usual animal tests. Further complications arose, too, from the fact that the assumption of identity of the two factors caused several workers to be side-tracked from the study of the vitamin requirements of yeasts to the study of the synthesis of vitamins by these organisms. This aspect of the problem is discussed in Section V.

Among the first observations that cast any doubt on the soundness of Williams's method were those of Lumière (1920). This observer showed that vitamins can be separated from the factors which stimulate yeast growth on the basis of (a) thermolability, (b) solubility in alcohol, (c) precipitability by phosphotungstic acid, and (d) adsorbability on fuller's earth. These differences between the two factors have been amply confirmed, e.g. by Fleming (1921), Goy (1921), Funk and Dubin (1921), Funk and Paton (1922), Freedman and Funk (1922 a), Willaman and Olsen (1923), Hosoya and Kuroya (1923 b), Peskett (1926). Some of the discrepancies which have been noted with the use of phosphotungstic acid can no doubt be elucidated in the light of the recent work of Kinnersley and Peters (1930), showing the influence of cH in the precipitation of vitamin B_1 phosphotungstates.

Since the subdivision of vitamin B into B_1, B_2, etc., little attempt has been made to identify bios with any of the B vitamins. Most of the experiments to which reference has just been made serve to differentiate it from what is now known separately as the antineuritic vitamin (B_1). In many respects bios resembles vitamin B_2 (Goldberger, Wheeler, Lillie and Rogers, 1926; Chick and Roscoe, 1927) and the factor for *Streptothrix corallinus* (Reader, 1928). The resemblance is

superficial, however, as many of the most potent antineuritic preparations containing no B_1 were found by Peters, Kinnersley, Orr-Ewing and Reader (1928) to possess bios and *Streptothrix* activity, and others showed no bios activity though presumably the *Streptothrix* factor was present (Peskett, 1929, unpublished data). The earlier work of Hoet (1922), unlike most of its time, is sufficiently precise to justify a similar conclusion. He used mainly a fermentation test for bios, but in some cases the yeast crops were weighed in addition. Hoet found that a *Penicillium* had a high content of bios but neither growth-promoting properties for rats nor curative nor protective properties for pigeons receiving a diet deficient in vitamin B.

Although it is thus probable that bios is neither vitamin B_1 (see also Guha, 1931), nor B_2 (see also Narayanan, 1930), the possibility of its being a vitamin-like factor necessary for a mammal or bird is not excluded. In the author's opinion, nothing but confusion will result if we accept misleading statements (see Section III) such as: "Bios, which is not essential for the growth of yeast but at most merely stimulates it, is therefore not a vitamin" (Pryde, 1931). Tanner (1925) takes a similar point of view to Pryde. Williams and Roehm (1930) consider that while Wildiers' bios has no relation to antineuritic vitamin, in the case of yeasts which (unlike Wildiers' yeast) are stimulated by a nutrient adsorbable by fuller's earth one of the substances involved may be the antineuritic vitamin. Using a seeding of 53,000 cells per c.c. they could detect the effect of adding 1 part in 100,000,000 of the crystalline antineuritic vitamin of Jansen and Donath (1927). At the same time it appears to be of little use to attempt to draw any conclusions as to possible relationships between bios and vitamins until one or other of the active principles is isolated in pure form.

To return to the bacterial factors: working with *Staphylococcus aureus* and in some cases with *B. dysenteriae* (Shiga), M'Leod and Wyon (1921) tested the potency of various materials as stimulants of growth. On the whole, good agreement was noted between the growth-promoting activity and the known "vitamin B" content of the various extracts, but certain important exceptions were noted in comparing the chemical and physical properties of the growth stimulant of yeast extract with those of "water soluble B." These differences were observed in the effects of alkali, heat and fuller's earth treatment. Bran and milk were exceptionally low in growth-promoting power in spite of their known content of "vitamin B." In the case of meningococcus and pneumococcus these authors stated that the growth-promoting power of various materials bore little definite relation to the known vitamin content. Suspended charcoal showed an appreciable effect as a growth-stimulant though "certainly devoid of vitamin." The pneumococcus factor was slightly thermolabile, especially in alkali, while that for meningococcus was thermostable. Both factors were destroyed by peptic or tryptic digestion; neither could be extracted from serum by means of 95 per cent. alcohol. The details recorded are scant, but sufficient to indicate that the growth factors of these four organisms are not identical with "vitamin B."

Mueller (1922 *a*) suggested that the substances supplied by meat infusion which

allow the growth of pneumococci are probably not vitamins, since muscle tissue is not a good source of the latter; which statement the later work of Hoagland (1929) confirms. Moreover, protein-free milk, which has long been known to contain vitamins (Hopkins, 1912) cannot replace meat infusion in the cultivation of pneumococci or *Streptococcus hemolyticus*.

Hosoya and Kuroya (1923 *a*) showed that the growth of most organisms was improved by addition of extract of vitamin B, but could proceed for a long time without any such addition. Thus the vitamin extract was not essential for *B. coli communis*, *V. cholerae*, *B. pyocyaneus*, *B. anthracis*, *B. typhosus*, *B. paratyphosus* A, *B. dysenteriae* (Shiga), *Staphylococcus albus* and *citreus*, *B. tetani*, *B. oedematis maligni* and *B. capsulatus welchii*. If these organisms require growth factors they are apparently able to synthesise them; such factors would not be vitamins *sensu strictu*. Further, in the case of meningococcus and *B. diphtheriae*, scanty growth was obtained in the absence of vitamin extract: *Streptococcus haemolyticus* and pneumococcus on the other hand could not be grown successfully without it. Hosoya and Kuroya (1923 *b*) made a further study using the same vitamin extract (Tsukie, 1922) which they considered to be essential for *Streptococcus haemolyticus*. It was also observed to stimulate growth as well as fermentation of yeast. These authors showed that for these two organisms the factors concerned behaved differently towards heat in alkaline solution (100° C. $N/40$ NaOH for 2 hours), and at the neutral point (182°–185° C. for 2 hours), the yeast factor being stable, the streptococcus factor being destroyed under these conditions. The antineuritic potency of the vitamin extract (tested on fowls and pigeons) was destroyed by heat at the neutral point (140° C. for 2 hours). The yeast factor was not adsorbed on fuller's earth, whereas the antineuritic vitamin was adsorbed. Both factors were shown to be adsorbed by animal charcoal from neutral solution. From this evidence the authors concluded that the substance required for the growth of the streptococcus is neither identical with bios nor with the antineuritic vitamin B. Moreover, the behaviour towards fuller's earth distinguishes the last two as different factors. These results are significant and the experiments seem to have been designed to meet most criticisms. The test of yeast-growth stimulation showed production of crops of about 100,000,000 cells from fairly small seedings (approximately 20,000 cells), though it is unfortunate that fermentation tests were conducted and described as confirmatory of bios activity. It is also noteworthy that nowhere do these authors state explicitly that the vitamin extract was essential for yeast growth, but they seem to conclude that they were dealing with Wildiers' "bios." This conclusion is doubtful (see Section III). In the proof of destruction of the streptococcus vitamin by heat, it was shown that the cultures usually, though not always, retained their vitality. Thus the possibility of a latent toxicity confusing the result was excluded to a large extent. Davis (1932) states that alkali-treated yeast extracts are strongly toxic to a lactobacillus which he has studied. Hosoya and Kuroya suggest that their factors are similar, but different, chemical substances and that yeast possesses greatest synthetic powers, and thus its factors are the simplest and most stable. Haemolytic streptococci being more fastidious require a less stable factor, while

the pigeon may require the most labile type of vitamin for growth and health.

Wyon (1923) pointed out the importance of amino acids as growth stimulants in testing the supposed activity of vitamins. Keeping the concentration of amino acids constant, he was able to show slight improvement of growth in the case of various bacteria by addition of either of two vitamin concentrates. The organisms which exhibited this stimulation were: *Staphylococcus albus* and *aureus*, *B. typhosus*, *B. paratyphosus* B, *B. dysenteriae* (Flexner), *B. prodigiosus*, *B. subtilis*, *Streptococcus haemolyticus*, a non-haemolytic streptococcus and a saccharomyces. The principal interest of this work lies in the observation that hydrolysed casein or meat extract, both, according to Wyon, containing little vitamin B, were as potent as the vitamin extracts when employed in equivalent concentration on the basis of their amino acid content. This behaviour suggested that, although the simple amino acids may not have been the active agent, polypeptides probably were, or substances such as thiovaline (Mueller's methionine). As regards the mode of action of the growth factors, Wyon inclined to the view that they acted by removal of inhibitory substances, just as catalase of blood removes peroxide.

Later, Goy (1925) published results which seemed to show conclusively that bacterial factors are different from vitamin B. He noted an improvement in the growth of *Aspergillus niger* on addition of extract of *Mucor mucedo*, likewise in the case of brewer's yeast on addition of extract of *Amylomucor β*. The active principle resembled vitamin B in so far as it was organic, dialysable and soluble in water and alcohol. It was destroyed by exposure to radium but stable at ordinary temperatures and at 130° C. (1½ hours) in acid solution. It acted in infinitesimal quantities and could not be replaced by gelatine nor could its activity be related to its amino acid content. Certain data, on the other hand, served to separate it clearly from vitamin B, for it was soluble in ether (see, however, McCollum and Simmonds, 1918; Williams and Waterman, 1926), not precipitated by phosphotungstic acid, and heat stable (1½ hours at 130° C.), when the reaction was neutral or alkaline. In addition the factor was found to be present in polished rice which is known to lack vitamin B and, according to Wollman (1921), *Amylomucor β* (Delemar) from which the active material was obtained, does not contain vitamin B. Growth of other organisms in addition to yeast was favoured by extracts of *Amylomucor β*, viz. staphylococcus, streptococcus, *V. cholerae*, *B. dysenteriae* (Shiga), *B. perfringens*, *V. septique*, *B. tetani*, *B. tuberculosis*, *B. sporogenes*, *B. histolyticus* and *B. diphtheriae*. It should be noted that Goy remarked that the control cultures (without growth factor) eventually attained the same crop as those which contained growth stimulant (cf. Lepeschkin, 1924). No details are given, but it seems reasonable to infer that these growth stimulants were not essential to the majority of organisms which he studied.

The case of *M. tuberculosis* requires special mention. This organism seems to have received less attention than most from the vitamin standpoint. Sazerac (1920) claimed to have obtained good cultures of both human and bovine strains, using a medium which contained autolysed baker's yeast. Long (1922), however, ob-

tained no improvement in growth with commercial yeast vitamin preparations, and Goy's work (1925) shows that extracts containing no vitamin B can stimulate growth. Uyei (1927 *a*) has made a further study of this organism. He claims that addition of 1:1000 Harris yeast vitamin to Long's medium causes stimulation of, and is essential for, growth, and suggests forcibly that the vitamin is the active agent. His results showed in fact some stimulation of growth (in both human and bovine strains with 1:100 Harris vitamin, not 1:1000); the active agent was found to be sparingly soluble in 90 per cent. alcohol and the control cultures with no vitamin exhibited moderate growth. These results suggest that the active agent was not vitamin B and not essential. From the evidence available it seems that *M. tuberculosis* falls into line with the majority of other organisms as regards vitamin B.

The doubtful significance of vitamin B as a growth factor for lower organisms is further supported by the work of Werkman (1927). In the first place Werkman pointed out that any work on these lines may easily be vitiated by the synthesis of the vitamin by the organisms themselves, which frequently occurs (see Section V). Secondly, observing the multiplication rates of *A. chroococcum* and *R. legumi-nosarum*, it was found that addition of vitamin B concentrate (Harris) caused acceleration of growth in the initial phases, but that in later phases the generation time was unaffected. This result is suggestive of oxidation-reduction effects. Werkman concluded that the concentrate acted as no more than a source of available food material, since a true vitamin effect should include catalysis of growth during the later as well as the initial phases. It should be pointed out that in these experiments the proof that the vitamin had not all been used up by the initial growth phase was not entirely convincing. Werkman also showed that alcoholic extract of the vitamin concentrate did not stimulate growth in concentration equivalent to 1:10,000, whereas the original material did, and that the alcohol-extracted concentrate behaved as the unextracted when tested in equivalent dilution. Vitamin B, on the other hand, can be extracted easily from such a concentrate by means of alcohol. Werkman's conclusions are in the main supported by the later work of Thompson (1929).

It will be seen that the foregoing evidence can be summarised as follows. Nearly all the organisms that have been studied—covering a wide range of species— responded by increased growth to addition of extracts containing "vitamin B." With the definite exception of the haemolytic streptococci and the pneumococcus and the possible exception of the meningococcus, *B. diphtheriae* and yeasts, the addition of such extracts was not essential. In the latter group *Cellulomonas folia* may also be included (see Section III). The supposition which can be found in some of the work that water-soluble vitamin B was the active agent in the extracts is practically unsupported by the experimental evidence, in fact much of the evidence is strongly against it.

As is generally known, "vitamin B" has now been subdivided into various fractions B_1, B_2, etc. Much of the above discussion loses, thereby, its specific interest, but the more recent work on bacterial stimulation by vitamins has been

amplified to some extent along these lines. Thus Reader (1928) found that *Streptothrix corallinus* responded to additions of antineuritic (B$_1$) vitamin concentrates with greatly improved growth. A quantitative "Streptothrix test" for vitamin B$_1$ was elaborated in which measurement of the growth stimulation of *S. corallinus* was used to assay the vitamin (Orr-Ewing and Reader, 1928 *a*). The method is liable to give anomalous results in the case of the cruder vitamin concentrates, although it takes into account the inhibitory effect of substances such as metals. For better preparations, only 1/80,000 pigeon unit in 20 c.c. was needed for marked stimulation. In an extension of this work to experiments on the isolation of vitamin B$_1$, Peters, Kinnersley, Orr-Ewing and Reader (1928) confirmed the finding of Reader (1928) that, whereas heat in alkaline solution inactivated the antineuritic vitamin, it left intact the factor for *S. corallinus*. However, the very close parallelism which these authors obtained between the pigeon and Streptothrix tests suggests that the factors concerned, though not identical, are probably closely related substances. Peters (1930) inclines to the view that Streptothrix actually needs torulin and can reactivate "alkalised" torulin. Many of the most potent antineuritic preparations which were active in stimulating growth of Streptothrix did not contain the thermo-stable factor (B$_2$) for rat growth. Thus B$_2$ seems to be excluded as a factor in the growth of Streptothrix.

Davis and Golding (1930) concluded that the factor which was necessary for luxuriant growth of a lactobacillus was not identical with vitamin B$_1$. The organism responded differently to various B$_1$ preparations of approximately the same potency for rat protection, and certain peptones which contained negligible quantities of B$_1$ as judged by rat growth, allowed good growth of the organism.

Mention must be made of attempts that have been made from a quite different aspect to settle the problem of the relation of vitamins to growth factors of lower organisms. The first of these was published by Heaton (1922) who studied the distribution of bios among the organs of normal animals and of animals suffering from deficiency of vitamin B. Unfortunately stimulation of fermentation and not of growth was used by him as the criterion for measuring bios activity in the results which he published (see Section III, pp. 21, 22). He found that all the organs of the pigeons which he examined showed approximately the same power to activate yeast fermentation, which contrasts strikingly with Cooper's results (1914 *a*) for the comparative value of the various organs in preventing polyneuritis (*i.e.* content in vitamin B$_1$). Heaton also found that the activating power of the organs was only slightly lessened in the case of pigeons suffering from polyneuritis owing to lack of vitamin (presumably B$_1$), but more markedly diminished in the case of rats which had been fed on a purified diet which caused cessation of growth. At the time his results were interpreted as showing that bios + antineuritic vitamin together form a complex necessary for rat growth, whereas the antineuritic vitamin alone is involved in the cure of polyneuritis. Later Thjötta (1924) found that the influenza bacillus grew as well on media containing blood which had been taken from birds suffering from polyneuritis due to lack of vitamin B as on media containing normal bird's blood. Similar observations

were made by Kollath and Leichtentritt (1925). At the present time a repetition and extension of work of this character seems highly desirable, using the more precise methods which are available for testing for bios, bacterial factors and the various B vitamins.

In conclusion several workers have reported that extracts known to be rich in vitamin C act as growth stimulants for certain organisms, *e.g.* influenza bacillus (see Section II), Yeast (Williams, 1921; Wright, 1922), tubercle bacillus (Uyei, 1927 *a*). Kollath and Leichtentritt (1925) found that the blood of scorbutic animals did not favour culture of influenza bacillus as did that of normal animals. These results could be equally well explained by assuming the formation of an inhibitory factor as by the disappearance of vitamin C as the active factor. In the case of organisms of the colon-typhoid type the work of Sugimoto (1931) suggests that vitamin C is not specific in this connection, but can be replaced by citrates. There is thus a faint suggestion that vitamin C is a possible factor in the growth of certain lower organisms, but any such action of the antiscorbutic vitamin remains as yet unproven.

V. GROWTH FACTORS PRODUCED BY LOWER ORGANISMS.

SUMMARY.

In view of the wide distribution of the factors which stimulate growth of bacteria, as can be seen in Section III, it is not surprising to find that they can in many instances be obtained from other bacterial species, and even from members of the same species. Such production of bacterial growth factors by bacteria is the probable explanation of some of the phenomena of symbiosis and "giant colony" formation as well as other less clearly defined growth effects which occur in mixed cultures—phenomena, the discussion of which is beyond the scope of this review. The amount of precise work that has been done on the subject is relatively small, and beyond a general statement that, in the case of a given species, growth may be enhanced or sometimes inhibited (see Barnes, 1931) by the presence of other living bacteria or their extracts, there is often little else to be said. For a general account, the reader is referred to Sergent (1928, pp. 47–54). The production of the X and V factors required by the influenza bacillus is mentioned below, but a fuller account will be found in the Medical Research Council's *System of Bacteriology* (1929).

The production of growth factors by yeasts deserves special mention. Extracts of yeast have been used very frequently as a ready source of growth factors (1) for lower organisms, (2) for yeast itself, and (3) for higher animals. As regards (1), Sergent, (1928, pp. 44–6) cites several instances, and other references to it will be found throughout the present article. As regards (2) and (3), the production of bios, the factor essential to the normal development of some if not all species of yeast, by yeasts themselves, has been one of the mysteries of the bios problem throughout its history. The fact that yeast contains all the known components of the vitamin B complex is well known, but there has been much controversy as to

whether these have been elaborated by the organism or have been acquired by it adventitiously, *e.g.* by adsorption. Most of the work that has been done on the synthesis of bios and vitamin B by yeasts receives notice in the review of Tanner (1925) which appeared before the subdivision of vitamin B. Some further details are given below.

For the purposes of this article the term "growth factor" has been used in a widened sense to include some of the vitamins of which the function is not exclusively growth-promoting, *e.g.* antineuritic vitamin B_1. Regarding the production of vitamins by bacteria, there is a good deal of precise information available probably on account of the more precise state of our knowledge regarding these factors. This subject does not appear to have been discussed in its entirety elsewhere. Briefly it may be said that much of the earlier work failed to show definite production of vitamins as the amount of organisms given to the test animals was too small. This was probably the case in the examples cited by Sergent (1928, p. 124). As a result of feeding larger amounts, there is now definite evidence that many widely separated classes of organism do produce vitamin B, though the amount may be relatively small. The biological significance of bacterial synthesis of vitamins is mentioned below, together with its bearing on the problem of "refection" (see Kon, 1931).

In experiments designed to test the ability of an organism to synthesise a given factor, certain points of technique are important, viz.:

(1) The choice of method used in testing for the factor; (2) adequate testing to prove the absence of the factor from the medium used for growth of the organism; and (3) avoidance of the effect of possible inhibiting factors (see Section II). So far, little attention has been paid to these details in respect of the factors for lower organisms, hence the information gained has been largely empirical. In the case of the production of vitamins, on the other hand, more precise work has been attempted since the earliest days, and as a result of careful control of the amount of organism used in the tests, it has now become possible to draw definite conclusions.

A. FACTORS FOR LOWER ORGANISMS.

Some examples of the production of growth factors by lower organisms, for the same or a different species, have already been discussed, but the following additional observations, which receive little or no mention in the articles referred to in the foregoing summary, may be cited here. Few in number as they are, they illustrate the widespread nature of the activities of lower organisms in this respect, and at the same time, the lack of precise information on the subject which must continue to exist until more definite knowledge is available of the exact nature of the substances involved.

Factors for acid-fast organisms.

Twort (1910) succeeded in obtaining good growth of the lepra bacillus and Johne's bacillus on media which contained the dead bodies of tubercle bacilli

(*M. tuberculosis*). He admitted (1931) that later the lepra cultures died out while the Johne cultures continued to do well. Twort and Ingram (1912) used media containing extracts of tubercle bacilli for cultivation of Johne's bacillus, and showed that other acid-fast organisms (*e.g. B. phlaei*) could supply the necessary growth factor, but that *M. tuberculosis* (bovine) contained very little. They also examined the solubilities of the factor (for Johne's bacillus) in alcohol and in chloroform, but in this respect their results appear to be in some cases contradictory. After cultivation for more than four months, Johne's bacillus could be acclimatised to grow without the addition of the factor to the medium so that some doubt must be entertained of its essential nature. Twort and Ingram (1914) also showed that certain vegetable tissues contained the necessary factor, while extracts from animals whose food consisted largely of these same vegetable materials, gave entirely negative results. Of the definite substances tested, glycerinic acid alone gave indications of a positive result. As regards *M. tuberculosis*, Uyei (1927 b) noted that small inoculations (10 mg. per c.c. or less) failed to grow whereas larger inoculations (50 mg. per c.c.) grew moderately on Long's medium. His attempts failed to demonstrate the presence of a growth factor either in cells of the organism (by extraction) or in their metabolic products (as in old tuberculin). Borrel, Boez and de Coulon (1923), however, had previously produced active extracts of the organism, and it may be noted that Goy (1925) showed that growth could be improved by an extract of *Amylomucor β*.

Factors for haemophilic organisms.

Thjötta (1921) claimed to have grown *H. influenzae* on media free from haemoglobin or its derivatives, but containing an emulsion or extract of Friedländer's bacillus or *B. proteus*. It was assumed that these organisms supplied the necessary factors. In a later communication, Thjötta and Avery (1921 a) admitted that such cultures of *H. influenzae* died after several generations, and suggested that two factors are required of which the bacterial extracts contained only one (see Section III). Damon did not confirm these findings (1923 a). In support of Thjötta, Hammerschmidt (1921) observed that *B. diphtheriae*, staphylococci, pneumococcus and Friedländer's bacillus would allow growth of Koch-Week's bacillus or influenza bacillus, when grown symbiotically. He noted a difference between the last two organisms in that *H. influenzae* grew best when the associated bacteria were living, while with Koch-Week's bacillus the best results were obtained with bacteria that had been killed by heating for 1 hour at 60° C. This difference contrasts with later work by Fildes (1923), Knorr (1925 b) and others, emphasising the close similarity of the nutritive requirements of the two organisms. Kollath and Taubmann (1928) studied the growth factors contained in a water extract of fresh plants. The dried crude extract ("phosphocrescin") was found to increase the production by bacteria of the *V* factor for the influenza bacillus. Lecithin had a similar effect to "phosphocrescin."

Factors for soil organisms.

Sanborn (1926 *b*) studied the effect of various organisms on the growth and physiological efficiency of *Cellulomonas folia. Actinomyces colorata, B. mycoides, B. subtilis* and *B. cereus* were all capable of stimulating this organism, when added either as living cells or as extracts. In the case of *Actinomyces, C. folia* lived at its expense so that ultimately the former died out. Associative stimulation was also demonstrated in the presence of living Azotobacter cells, though in this case potent extracts could not be prepared. Later, Sanborn and Hamilton (1929) showed that preparations of the gum of Azotobacter stimulate growth and fermentation of *C. folia.* The gum is precipitated by normal lead acetate, and resembles that which Buchanan (1909) prepared from *Rhizobium.*

Factors for Streptothrix corallinus.

Orr-Ewing and Reader (1928 *b*) found that a meat extract medium which had been freed from *S. corallinus* growth factor by repeated charcoal extraction was capable of supporting good growth of this organism after a strain of meningococcus had been allowed to grow in it for six days. This affords an interesting example of the production of a growth stimulant by one of the more delicate bacterial species, which itself needs at least one factor for abundant growth (see Section III).

Factors for fungi.

Unless the sugar or tartaric acid of his medium were contaminated with bios, the work of Hoet (1922) can be interpreted as proving that the species of *Penicillium* which he used was able to synthesise bios. Schopmeyer and Fulmer (1931) have demonstrated the production of a yeast growth stimulant by various moulds: *Aspergillus niger, Aspergillus clavatus* and *Trichoderma lignorum,* but their published data refer only to *Aspergillus niger.* Various recognised breakdown products of sucrose and glycerol, which were used as components of their medium, were tested for yeast stimulation and found to be inactive. The synthesis of bios by yeasts is discussed fully by Tanner (1925).

B. FACTORS FOR HIGHER ORGANISMS (VITAMINS).

One of the earliest observations was that of Cooper (1914 *b*) who showed that extracts of *B. coli* do not possess antineuritic properties for pigeons. On the other hand, Pacini and Russell (1918) showed that extracts of cultures of *B. typhosus* contained a growth-promoting substance for rats receiving a diet deficient in vitamin B. The organism had been grown on Uschinsky's medium, which was tested and found to contain no vitamin. When the test animals had reached constant weight (14 days on the basal diet) the extracts were added to the diet in amounts equivalent to less than 5 gm. of the organism per day.

Damon (1921) tested various bacteria for their ability to produce the growth-promoting factor for rats. The killed bacteria were taken up on starch and fed as

supplements to the diet, in the case of *B. paratyphosus* B and *B. coli*, 600 mg. per 100 gm. ration and in the case of *B. subtilis*, 20 gm. per 100 gm. ration. All failed to check the loss in weight of the rats. In a later communication (1923 *b*) he tested three types of bacteria. Five per cent. of Friedländer's bacillus or of an aerobic spore-forming bacillus (*B. adhaerens*) in the diet had no effect on the growth. Another mucoid organism (Pfeiffer's bacillus) on the other hand maintained the animals at a constant weight or induced rapid growth, as did an acid-fast organism (*B. phlaei*) when fed 5 per cent. of the diet—10 per cent. in the diet caused rapid and continuous growth. With the exception of the last-named organism, all the bacteria had been grown on a medium said to have been made up of components of known composition or tested to show that it contained no vitamin. At the same time one is led to question the purity of a medium which apparently permitted profuse growth of such a fastidious organism as Pfeiffer's bacillus. The *B. phlaei* had been grown on veal-peptone broth which might have contained vitamins. Subsequently (Damon, 1924) various acid-fast bacteria (*B. smegmatis*, *B. phlaei* and *B. Moelleri*) were grown on a medium which contained peptone, beef extract and glycerine, and shown to produce normal growth curves in rats receiving a deficient diet which contained 7·5 per cent. of the organism. At the level of 2·5 per cent. a decline in weight occurred and polyneuritis supervened after 8 weeks. It had been shown in the earlier work that the peptone and beef extract did not contain water-soluble vitamins.

Weill, Arloing and Dufourt (1922) reported that the feeding of a mixture of organisms isolated from pigeons' faeces did not prolong the period of survival of pigeons receiving a diet of polished rice. The amount of organism fed appears to have been extremely small ("0·05 to 0·10 cgr." dried organisms).

Kuroya and Hosoya (1923) showed that *B. coli* which had been grown through seventy generations on Fraenkel's medium contained substances which permitted growth of streptococci, pneumococcus, meningococcus and *B. diphtheriae* on the same medium with addition of 0·5 per cent. glucose. They claim that extract of *B. coli* stimulates also the growth and fermentation of yeast. This was confirmed by R. C. Robertson (1924), though it should be pointed out that Kuroya and Hosoya's figures as tabulated show exactly the reverse. The factor was found to withstand heat for 2 hours at 140° C. except in alkaline solution (*N*/10 NaOH). Since administration of the organisms cured polyneuritis in pigeons and the extract allowed growth of rats on a deficient diet, it was assumed that *B. coli* could synthesise bios, vitamin B and streptococcus vitamin. From their figures it may be calculated that extract equivalent to 0·018 gm. of dried organism was fed per rat (40–70 gm.) daily, whereas the pigeons (255–295 gm.) received 0·5 gm. dried organism per bird daily. The weight of the pigeons receiving the organism was not improved. Heller, McElroy and Garlock (1925) reported that some spore-bearing intestinal organisms contain vitamin B.

In the same year as Kuroya and Hosoya, Scheunert and Schieblich (1923) reported that *B. vulgatus* (Flügge) Migula could synthesise vitamin B, as judged from cure of polyneuritic pigeons, and that the weight of the birds improved if sufficient

of the organism was fed (3 gm. dried organism to bird weighing 190 gm.). Later (1927 *a*) these authors showed that the same organism could synthesise the growth-promoting vitamin B for rats, and studied the effect of *p*H of the culture medium on this synthetic process. These results have been amply confirmed, first by Scheunert and Schieblich (1927 *b*) in answer to criticisms raised by Bieling (1925), who questioned the freedom from vitamin of the medium used for culture of the organism, and later by Schieblich (1929, 1930). In the last paper Schieblich (1930) made a comparative study of the vitamin synthesis of *B. vulgatus* (Flügge) and *B. mesentericus* (Flügge): the latter organism showed less synthesis than the former.

Hunter (1923) claimed to have shown that Azotobacter could synthesise "a factor similar to vitamin B." The organism was grown on a medium of which the only component whose purity may be doubted was the dextrose. In a preliminary experiment, rats fed on a vitamin-free diet showed some improvement in growth when 2 per cent. of the air-dried organisms was added to it, though the growth was very poor (5 gm. gain in weight per week). In later experiments, rats grew equally well on a similar diet when 2 per cent. of the dried organisms or 2 per cent. of baker's yeast was added. Here the gains in weight were not more than 25–30 gm. in 20 days. One is led to question the significance of these results as the diet contained 10 per cent. "tankage," and the weight of the animals on the yeast diet declined later. An explanation of this decline may be found in the work of Williams, Waterman and Gurin (1929) and Evans and Lepkovsky (1929) showing baker's yeast to be a less potent source of vitamin than brewer's, though in the work of Kennedy and Palmer (1922) the reverse seems to have been the case. Hunter's results in any case show that some vitamin B_1 synthesis occurred, for 1 gm. of dried organisms alleviated polyneuritis of pigeons for 2 to 3 days.

It may be noted that Mockeridge (1924 *a*) reported that *Azotobacter*, when grown upon an agar medium, produces substances capable of stimulating plant growth—"auximones." Since various materials which contain auximones had been found to contain nucleic acid radicals, *e.g.* baker's yeast and *B. radicicola* (Bottomley, 1919), Azotobacter (Mockeridge, 1924 *b*), it was suggested by Mockeridge that auximones had nucleic acid as a basis. According to the work of Mazé (1919) and Lumière (1921) such auximones are not in all cases essential for plant growth. Jensen (1931) records the production by micro-organisms of a substance promoting growth of *Avena* coleoptiles.

Sunderlin and Werkman (1928) made a thorough study of the production of vitamin B by various organisms. Their paper contains an excellent though very brief résumé of the literature and a bibliography which includes some references not quoted in the present discussion. Rats were used as test animals. The organisms were mostly fed separately and had generally been grown on a synthetic medium. In any event, the medium was tested as a control. Sunderlin and Werkman point out that much confusion has arisen in the earlier work owing to (1) disregard of the definition of the term "vitamin," (2) wide variation in experimental technique in testing for vitamin. The organisms were fed in comparatively large quantities

(up to 8 gm. per rat per day) during several weeks. Under these test conditions widely separated species were found to synthesise vitamin B (growth-promoting for rats), namely *Torula rosea, Oidium lactis, B. adhaerens, B. coli, B. subtilis, B. mycoides, Azotobacter chroococcum, Rhizobium leguminosarum* and an *Actinomyces*. It may be remarked that Hoet, Leclef and Delrue (1924) had tested *Monilia candida, Torula rosea* and *Mycoderma* which had been grown on synthetic media. They found that *Monilia* alone of these organisms contained vitamin B as judged by its curative action on polyneuritic pigeons receiving extract of 2–3 gm. daily, and its growth-promoting property for rats (receiving 0·5 gm. dried organisms daily), although the other two organisms were fed to pigeons in larger quantities than the *Monilia*. As regards rat tests, *Torula* was fed at the level of 6 per cent. of the ration, while no record is given of rat tests of *Mycoderma*. These results are not incompatible with those of Sunderlin and Werkman if considered on a quantitative basis.

To complete discussion of the subject, some mention must be made of the general biological significance of the synthesis of vitamins by lower organisms. The vitamin A synthesised by marine diatoms and other organisms (Coward and Drummond, 1921; Hjort, 1922; Jameson, Drummond and Coward, 1922) is, in all probability, the ultimate source of the large quantities which are present in the liver oils of cod and other fish. The diatoms form the food of a number of minute animals which are eaten by small fish and other animals, the latter providing food material for cod and other larger fish. The ability of yeast to synthesise at least two of the components of the vitamin B complex (B_1 and B_2) is suggested by the work of Harden and Zilva (1921), MacDonald (1922) and many other observers. The question is discussed fully by Tanner (1925) who, however, omits the following observations. Darrah (1922) found only slight indications of the presence of vitamin B in yeast grown on media which contained extracts of cereals, the vitamins of which had been destroyed. Heller (1923) showed that *S. cerevisiae* synthesised both growth-promoting and antineuritic vitamins when grown on synthetic media, though such yeast is not so rich in these factors as that grown on wort. It was noted, too, that even when the former yeast was fed at the level of 5 per cent. of the diet, the young rats, shortly before weaning, did not develop normally though their mothers had grown and developed normally. More recently the conclusion of Hawking (1927) was supported by Peskett (1927 *b*) who showed definite synthesis of antineuritic vitamin by *S. cerevisiae* grown in absence of bacterial contamination. The synthetic production of vitamins A and B by these organisms is of particular interest as fish-liver oils and yeast respectively are among the most potent sources of these vitamins known.

It must be recorded here that certain higher organisms seem to be able to develop normally on a B-deficient ration. This was reported in the case of cattle by Bechdel, Eckles and Palmer (1926), who showed also that on the deficient diet milk was produced, the vitamin B content of which, as judged from rat growth tests, was not markedly reduced. The latter observation was confirmed by Bechdel and Honeywell (1927). The ultimate source of the vitamin was considered by

Bechdel, Honeywell, Dutcher and Knutsen (1927) to be a micro-organism, *Flavo-bacterium vitarumen*, inhabiting the rumen of cattle. These authors (1928) claim to have proved that this organism can synthesise vitamin B when grown on a medium which they tested and found to be free of vitamins. From their data it is difficult to determine exactly how much of any vitamin originally present in the medium is represented by the 0·5 gm. "residue" which was fed: it appears that this represented approximately 50 c.c. medium. Their figures show an average weekly gain in weight of 6·8 gm. in rats receiving daily 0·5 gm. of the dried organism. So far no confirmation of their work appears to have been published. The original suggestion of the hypothesis of a synthetic production of vitamin B in the alimentary tract was made by Theiler, Green and Viljoen (1915).

A similar synthesis of vitamin B by bacteria has been postulated to explain the phenomena of "refection." In this condition, first described in rats by Fridericia (1926), normal growth occurs in spite of a deficiency of vitamin B in the diet. A frequent symptom of the condition is the appearance of white or pale-coloured faeces containing large amounts of unchanged starch grains. Fridericia, Freu-denthal, Gudjonnsson, Johansen and Schoubye (1927) found that vitamin B is produced, since the faeces can be shown to contain appreciable quantities of vitamin by rat-growth tests (carried out with heated faeces). They also point out that the common intestinal organisms, though capable of production of vitamin B, cannot be responsible for refection or the condition of vitamin deficiency would not exist. For a full bibliography of the subject, the reader is referred to the article by Kon (1931). At present bacterial synthesis seems to be the most plausible explanation of the phenomenon.

VI. SUMMARY.

The foregoing article is an attempt to give a general account of the growth factors of lower organisms, *i.e.* the substances which must be supplied in addition to food materials of known composition in order that normal growth can occur. As regards the growth factors required by lower organisms, the number of cases to which such a strict definition can be applied is limited to a few, and it has been necessary to include discussion of factors of which the indispensable nature is uncertain. In the case of growth factors used by higher organisms, discussion has been limited to a consideration of vitamins which are all, by definition and common usage of the term, essential. In most instances the chemical composition of growth factors is unknown.

In order to give the biological reader as general a view as possible the author has included an account of some of the more important experimental details which may influence growth—not necessarily growth factors in the strict sense—though these should be already well known to specialists in the field under review. These influences may be connected with chemical substances in the culture medium, *e.g.* inhibitory substances, carbon dioxide, amino acids, salts; or with its physico-chemical properties, *e.g.* surface tension, pH, oxidation-reduction potential.

Further, a discussion is included of two important points connected with the organism itself, viz. size of seeding and cultural characteristics (Section II).

There is evidence for the existence of growth-stimulating factors in the case of most of the organisms which have been studied. Often proof has been lacking of the absolute necessity of such factors for growth, and it is true to state that much of the work that has been carried out, beyond showing that growth of an organism can be improved by addition of certain materials, has added little to our real knowledge of the problem. More detailed knowledge of indispensable factors has been obtained in the case of some of the Coccaceae, Bacteriaceae (tribe Haemophileae), Mycobacteriaceae, Chlamydobacteriaceae and Fungi. In the Bacteria (Schizomycetes) the organisms which have attracted most attention have done so on account of their medical interest, most of them being pathogenic. Effort has therefore been directed towards obtaining a simple method of culturing the organisms rather than solving the fundamental problems of growth which are involved. As a consequence much of the work is disjointed and cannot be co-ordinated in review (Section III).

The growth factors of lower organisms, especially those which are essential, are obviously similar to the vitamins required by higher organisms. Advance of our knowledge of vitamins has led to multiplication of the number of factors that are known to be required by animals. In spite of this and the increased information now available, none of these vitamins has been definitely shown to be identical with any of the factors required by lower organisms, though frequently the resemblance has proved to be very close (Section IV).

The question of production of growth factors by lower organisms involves (a) factors for higher organisms, and (b) factors for other lower organisms. It has been shown that production of both these types of growth factor is a widespread occurrence. A more difficult problem awaiting solution is mentioned, viz. the production of factors required for growth of a given species by that organism itself. This may occur in the case of yeasts, for example, which do not grow from a small seeding on a synthetic medium if bios is absent, though if a large seeding is used a sufficient crop may be obtained to show that bios is present therein (Section V).

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RECENT PROGRESS IN THE CHEMISTRY OF MUSCULAR CONTRACTION

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(With One Text-figure.)

CONTENTS.

I. INTRODUCTION.

COMPARATIVELY little is known about the mechanism of the muscles of invertebrates, and this review will of necessity deal mainly with the muscles of vertebrates and in particular with the muscles of the frog. Functionally a typical voluntary muscle is characterised by a rapidity of movement, ability to tetanise, and complete lack of tonus when separated from the central nervous system. Heart muscle is distinguished by spontaneous rhythmic activity, and an absolute refractory period of such length that complete fusion of successive contractions is impossible. Involuntary muscles, like the heart muscle, are primarily self-governing, but, unlike the heart, which exists only in two states, contracted and relaxed, the typical visceral muscle can maintain for indefinite periods a state of partial contraction. Indeed so much is this a property of such muscles that it is hardly possible to speak of the "resting" length of a strip of involuntary muscle in the sense in which the term can be applied to voluntary or cardiac muscle. An irregular spontaneous rhythm is sometimes displayed and the rate of movement is in general very slow.

Associated with these functional differences are well-marked histological differences (Denny Brown, 1929) and some definite differences in chemical composition; for example, the glycogen store of the cardiac muscle is very high, amounting sometimes to nearly a tenth of the total solids, and this store is maintained even in circumstances where the needs of the rest of the body for carbohydrate are urgent. The glycogen store of a skeletal (voluntary) muscle is found to vary considerably according to the nutritional state of the animal and the functional state of the muscle. Figures varying from a trace to rather more than 1 per cent. have been recorded. Involuntary muscles have not as a rule large stores of glycogen. Differences have been recorded in the cholesterol content of muscles of different types, in the hexosephosphate content and so forth. Leathes reported many years ago that red muscles of the rabbit contain more fat than white. Habs (1929) has lately found the glutathione content of heart muscle is four times that of white muscle, red muscle being intermediate (confirmed by Okuda (1930)). In the case of vertebrates there is a large difference in creatinephosphoric acid content as between voluntary muscles and muscles of the other two types. Roughly speaking, the voluntary muscle contains ten times as much as the cardiac or visceral muscle, and as much as half of its phosphorus may be in this form.

II. RESTING METABOLISM.

A muscle from a warm-blooded animal requires considerably more oxygen than it could obtain by simple diffusion from its surroundings except in very high pressures of oxygen; most work on isolated muscles has therefore been done on cold-blooded animals. The skeletal muscles of the frog use about 0·025 c.c. of oxygen per gm. per hour at room temperature (Meyerhof and Schulz, 1927), a quantity which can diffuse into a flat strip of muscle from air if the muscle be not more than 2 mm. thick, or from an atmosphere of pure oxygen if the thickness does not exceed 5 mm. (Hill, 1928 *a*). Measurements of the oxygen consumption of isolated tissues have sometimes been made in which this condition has not been observed, and a state of partial oxygen lack has vitiated the measurements. Carbon dioxide is formed in the muscle and its rate of formation has been measured by keeping the tissue in a closed vessel containing alkali or by passing a stream of oxygen over the tissue and bubbling this oxygen afterwards through alkali. A drawback to either method is the fact that the atmosphere must contain no carbon dioxide, for since most tissues are in equilibrium with about one-twentieth of an atmosphere of carbon dioxide they lose carbon dioxide on exposure to a carbon dioxide-free atmosphere. The formation of organic acids during partial anaeorbiosis will also tend to bring about the further loss of carbon dioxide. Measurements of the carbon dioxide rate tend therefore to be high and of oxygen consumption rate to be low. Estimates of the respiratory quotient are liable on both scores to be too high. The respiratory quotient of skeletal muscles and of cardiac muscle from the frog has been measured by several observers (see particularly Meyerhof and Schmidt (1929) and Fenn (1927)), whose results vary between 0·85 and 1·07 with an average of about 0·94.

The chemical reactions going on in the isolated muscle, including the combustion

processes indicated by the oxygen and carbon dioxide measurements, result in an evolution of heat. This heat production is very slight—about 0·2 cal. per gm. per hour (Hill, 1928 *b*)—and only recent refinements of thermometric technique have made its measurement possible. The combustions indicated by these measurements should result in the disappearance of carbon compounds from the isolated muscle, but the quantities are too small to detect by weighing. Something like 40–80γ of substance should disappear in 10 hours from a muscle weighing 200 mg. Now such a muscle contains about 35 mg. of protein, 2 mg. of carbohydrate and 1 mg. of fat. The metabolism over 10 hours is hardly likely to be detectable by direct analysis. It is not surprising that skeletal muscles of the frog have been maintained in good condition with adequate oxygen supply for several days. No chemical changes have been observed to occur in surviving muscles during the first twenty-four hours of survival if oxygen is abundant.

Complete deprivation of oxygen leads to comparatively rapid death of isolated muscle. The beating heart of a frog survives only an hour or two and the resting skeletal muscles about 36 hours, that is to say, at the end of this period administration of oxygen fails to bring about a return to normal or approximately normal conditions of the tissue. There is initially a production of carbon dioxide by the muscle, but this seems entirely attributable to the formation of acids which expel the pre-existing carbon dioxide. Resting muscles straight from the animal contain about 12 volumes of carbon dioxide per 100 c.c. of tissue (Stella, 1929). Heat is produced by the muscle at a steady rate, at least until an advanced stage of oxygen lack has been reached. This heat rate is about two-fifths of that characteristic of the muscle when fully supplied with oxygen. There have been observed two chemical changes which do not occur in the latter case. They are the formation of orthophosphate and of lactate. The production of lactic acid occurs mainly, if not entirely, at the cost of glycogen. The increase in the lactic acid content and decrease of glycogen content resulting from a few hours' lack of oxygen are found to be comparable within fairly narrow limits (Parnas and Wagner, 1914). The rate of lactic acid production falls off as time progresses, though a rough average value at room temperature is 0·15 mg. per gm. per hour, and generally ceases before all the glycogen of the muscle is used up. The amount of lactic acid which can accumulate in these conditions forms the basis of a distinction of different types of muscle, for it is observed that the accumulation is least in involuntary muscle and greatest in voluntary (up to 600 mg. per 100 gm.). The extent of lactic acid formation does not appear to be conditioned by the available glycogen, for cardiac muscle produces relatively little lactic acid post-mortem although it contains large glycogen stores.

It is more probable that the process of glycolysis is brought to an end by the acidity of the muscle. Lohmann (1926) finds that the lactate-producing enzyme system has only feeble activity in muscle extracts more acid than *p*H 6·5, and Kerridge and collaborators record the *p*H of muscles in rigor to be 6·0 (skeletal), 6·4 (cardiac) (Furusawa and Kerridge, 1927) and 6·6 (uterus) (Kerridge and Winton, 1929). The lactate concentration reached in rigor would on this view be a rough measure of the buffering power of the tissue.

Table I contains a summary of the "metabolic coefficients" discussed in this section.

Table I. *The metabolic rate of isolated muscles of the frog.*

	15°	20°
Anaerobic heat cal./gm./hour	0·057	0·092
Lactate production mg./gm./hour	0·09	0·16
Aerobic heat cal./gm./hour	0·14	0·23
Oxygen consumption mg./gm./hour	0·024	0·04

(These are averages of rather variable figures and intended only to convey an approximate idea of the magnitudes.)

The rate of phosphate production following deprivation of oxygen changes with time in an even more marked manner than the rate of lactate production. During the first 5 hours (at room temperature) it is rapid, and the phosphate gain is balanced exactly by the loss of creatinephosphoric acid (phosphocreatine, phosphagen). Later other phosphoric esters decompose, but the rate of phosphate production is greatly reduced (Eggleton and Eggleton, 1929). There is obviously a discrepancy here between the steady rate of heat production and the falling rate of these chemical reactions. The discrepancy suggests the existence of other reactions as yet undiscovered, and, as will be seen later, osmotic pressure measurements confirm this idea.

III. THE NITROGEN AND PHOSPHORUS COMPOUNDS IN MUSCLE.

The phosphorus compounds in the skeletal muscles of the frog appear to be few in number. The orthophosphate and phosphagen together represent rather more than half the acid-soluble phosphates of the muscle, and most of the remaining phosphorus is present as adenylpyrophosphoric acid (adenosine triphosphoric acid), a somewhat complicated purine derivative, the structure of which is not yet known in detail, but may be provisionally represented as

pyrophosphate

Adenine-ribose-orthophosphate
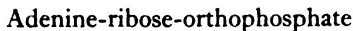
The small remainder of the acid-soluble phosphorus is nearly accounted for by the hexosemonophosphoric ester discovered by Embden and named by him lactacidogen (Embden and Zimmerman, 1927)[1]. The "acid-soluble phosphorus"—the phosphate remaining in solution when the proteins of a tissue are coagulated by means of trichloracetic acid or picric acid—is not quite all the phosphorus of the muscle. There is found in the protein precipitate a small quantity of phosphorus which is probably chiefly lipoid. This small amount appears to be distinct physiologically in that it is unaltered in amount by any treatment to which surviving

[1] The term lactacidogen was applied during the period 1912-27 to a hexosediphosphoric ester found in specially treated muscle extracts but now known not to be present in normal muscle.

muscles have so far been subjected (Eggleton and Eggleton, 1928; and Wechsel-mann, 1921). Its distinction from acid-soluble phosphorus was in the first place one of chemical convenience, but it appears to have a physiological justification. No one has yet succeeded in elaborating a system of analysis for the phosphorus compounds of muscle, but it is convenient to distinguish in the acid-soluble group between phosphorus derivatives with soluble barium salts (which include phosphagen and hexosemonophosphate) and those with insoluble barium salts, including orthophosphate and adenylpyrophosphate. Analysed in these terms, muscles show characteristic differences in composition according to their physiological state (Table II).

Table II. *Distribution of acid-soluble phosphorus in a typical skeletal muscle of a frog.*

	Resting	Rapidly fatigued	Dead
Orthophosphate	9	33	90
Phosphagen	54	24	0
Pyrophosphate	22	22	0
Esters* with soluble barium salts	4	10	10
Esters† with insoluble barium salts	11	11	0
Total	100	100	100

The total acid-soluble phosphorus amounts usually to 1 20–1 50 mg per gm
* Almost exclusively hexosemonophosphoric ester, in living muscle
† Almost exclusively adenylic acid (and in combination with pyrophosphate)

Not only is the "acid-soluble" phosphorus now practically all identified but the "acid-soluble" nitrogen (non-protein nitrogen) is largely accounted for by the known and measurable constituents. In Table III are assembled rough average values for seven constituents, which, it will be seen, leave only 4 per cent. of the total nitrogen to be traced.

Table III. *Distribution of total non-protein nitrogen in an average resting frog skeletal muscle.*

	mg N per 100 gm
Glutathione	2
Creatine	35
Carnosine	45
Phosphagen	120
Adenylic acid	35
Urea	12
Ammonia	1
Unknown	10
Total	260

This table is based largely on some as yet unpublished analyses performed by M. G. Eggleton and the writer, in which the total nitrogen and also the individual

compounds were estimated. The analyses are in good agreement with isolated observations in the literature—but it is notoriously unsafe to attempt to build a table out of results obtained by different workers on different material. (An example of this probably occurs in Table I, where the oxygen consumption rate of muscle, measured by Meyerhof on German frogs (*Rana esculenta*), and the aerobic heat rate measured by A. V. Hill on larger Hungarian species, are brought together. It will be seen that the calorific value of oxygen derived from the two figures is 8 cal. per litre, a value nearly twice as great as that characteristic of the oxidation of the ordinary foodstuffs, or indeed of almost any carbon compound. The apparent discrepancy will not improbably vanish when both measurements are made on muscles of the same individual frog.)

IV. DIFFUSION OF SUBSTANCES THROUGH MUSCLE TISSUE.

There can be little doubt that the ultimate ill effects of oxygen lack on an isolated frog muscle are due to the accumulation of the products of anaerobic metabolism rather than the disappearance of essential food supplies. If the muscle is small and thin, immersion in Ringer's fluid considerably improves its condition and post-pones death for some days. In such conditions there is a diffusion away from the muscle of a number of simple compounds among which lactate, creatine, phosphate, potassium and urea have been identified. Not all of these are necessarily deleterious to the muscle, though the removal of the lactate undoubtedly delays the onset of rigor, and the removal of potassium may in certain circumstances be necessary to permit the condition of excitability.

When the sartorius muscle of a frog is dissected without the use of Ringer's fluid, but with every precaution against drying and mechanical injury it nevertheless frequently becomes inexcitable in the course of a few minutes. Analysis of such muscles has so far revealed no abnormality, but since soaking in Ringer's fluid rapidly restores excitability, the possibility suggests itself that something harmful is diffusing away. The idea is favoured by the experience that if a small quantity of fluid is used to resuscitate several muscles in succession it loses virtue with each successive cure and ultimately fails to have a beneficial effect. It is then found to contain about four times its original content of potassium. A Ringer's solution made up with a fourfold potassium content is useless from the start and may even induce inexcitability in normal muscles. A gastrocnemius muscle rarely develops spontaneous inexcitability, but pricking with a pin will induce it, and subsequent immersion in saline will completely restore excitability.

These and other observations suggested to Horton (whose work is quoted above) that the potassium of these muscles is normally restricted to the fibres, and that if injury to a few fibres permitted potassium to escape into the interspaces excitability is reduced or even abolished. Only one fibre in two hundred need be supposed to lose its potassium in order to raise the concentration of that ion in the interspaces to 0·03 per cent. (four times normal Ringer concentration). This is no more than twenty fibres in an average sartorius muscle (see Horton, 1930; and Dulière and Horton, 1929).

The diffusion of certain substances from muscles has been studied in some detail

and a considerably better understanding of the structure of muscle tissue is likely to be gained by further work on the same lines. Lactate, for example, diffuses away from dead muscles at the rate at which it would escape from a lump of jelly of equal size and shape. No hindrance exists to its passage through or around the fibres. But from a living fatigued muscle it diffuses at only one-eighth the rate (its diffusion constant is one-eighth of the normal). It is as though the living cells were able to hinder the passage of lactate through them and the lactate had to escape mainly through the plasma filling the interspaces (Eggleton, Eggleton and Hill, 1928). The only other accurate measurements that have been made of diffusion through tissues appear to be those of Krogh (1918) who found the diffusion constant of oxygen through muscle to be half that through a 15 per cent. gelatin solution. A study of the rate of diffusion of dyes through living and dead muscles would probably contribute useful knowledge in this connection. Not only can the diffusion constant be measured but the apparent concentration of a diffusible constituent of muscle can be ascertained by determining the concentration of the substance in the surrounding fluid necessary to prevent·diffusion into or out of the muscle. Such experiments have been performed by Dr Devadatta who finds the concentrations of lactate in resting and fatigued muscles measured in this way to be the same as those measured by direct estimation (private communication to the writer). A similar conclusion was reached in respect of orthophosphate by E. Semeanoff (1931), although some earlier experiments by Stella (1928) gave results indicating that some of the directly estimatable orthophosphate was not osmotically active. Stella found the diffusion constant of phosphate through muscle tissue to be approximately the same as the value recorded for its diffusion through water.

The concentration of creatine in resting muscles (frog) estimated by the counter diffusion method appears to be about 80 mg. per 100 gm. (Eggleton, 1930). Figures of just this order have also been found by direct analysis (Dulière, 1929). The "total" creatine of voluntary muscle—estimated by the Jaffé reaction after boiling the tissue with acid—is a considerably larger quantity than this, about 400 mg. per 100 gm. But the major part of this is present in the resting muscle as creatine-phosphoric acid (phosphagen) which is incapable of diffusing out of the muscle. Two methods are available for the estimation of the non-phosphagen creatine, and since these agree with the estimates of diffusible creatine both in the case of resting and in the case of fatigued muscles it seems unlikely that a third form of creatine exists in such muscles.

It is not clear why only the free creatine and the free orthophosphate in a muscle should be diffusible, but such appears to be the case. Such compounds as hexosephosphate, adenosine triphosphate and creatinephosphate can diffuse quite well through collodion membranes although they cannot escape from the muscle (Rothschild, 1929).

For the purposes of biochemical estimations it is convenient to treat a tissue as a homogeneous lump of stuff, but it is becoming more and more necessary that attention should be paid to the actual distribution of any one constituent in a tissue. In the future science of histological chemistry the diffusion techniques outlined above will perhaps be one method of approach.

V. THE QUANTITATIVE STUDY OF HEAT, LACTATE AND PHOSPHATE PRODUCTION IN STIMULATED MUSCLE.

The stimulation of an isolated muscle is generally accomplished by the application of an electric shock either to the muscle itself or to its motor nerve. There is no evidence that the chemical changes accompanying the response are in any way different in the two cases.

Consider first the simplest case of an isolated muscle adequately supplied with oxygen, performing a long series of twitches. Very few experiments of this type have been performed, since it is nearly impossible to achieve adequate oxygen supply by diffusion for all but the smallest muscles. At one atmosphere pressure of oxygen (and greater pressures are hardly feasible when other necessary limitations are taken into account) a flat muscle could probably give a twitch every 10 sec. without becoming asphyxiated if it were not more than 1 mm. thick (A. V. Hill, 1928 *a*). It would take many hours at this rate to produce significant changes in its composition. From such published experiments as comply with these requirements it can be deduced that the only notable change consistently found to accompany the performance of work in these circumstances are the disappearance of carbo-hydrate, the absorption of oxygen and the formation of carbon dioxide. Dis-appearance of protein would be difficult to trace by direct estimation because of the large amount present, though the reported formation of ammonia in minute traces (Parnas and Mozolowsky, 1927) may be in part the result of a combustion of amino acids. A steady liberation of diffusible nitrogen compounds has been observed by Clark and his collaborators (1930) to occur in the surviving heart of the frog, beating in well-aerated saline. Several attempts to detect a disappearance of fat from the working muscle have failed (Winfield, 1914).

The chemical processes accompanying activity of a muscle are apparently always anoxidative, even when oxygen is present in abundance. The course of heat pro-duction during a twitch is just the same whether the muscle has sufficient oxygen dissolved in it or is completely lacking oxygen (Hill and Hartree, 1920). Not until some seconds after the twitch is over does a difference become evident between the two cases, when the oxygenated muscle begins a second outburst of heat lasting several minutes, and equal in amount to the heat associated with the twitch. The anaerobic muscle evolves little or no such delayed heat. On the average the amount generated during this phase is perhaps one-tenth that characteristic of the muscle saturated with oxygen. The heat evolution accompanying the twitch occurs in two

Approximate distribution of heat associated with single twitch of frog sartorius (in arbitrary units).

	During contraction	During relaxation	During subsequent 1–2 minutes
Oxygen absent	2	1	0–1
Oxygen present	2	1	3

batches, one coinciding with the contraction of the muscle, and the other with relaxation (Hartree, 1931).

It is possible to divide up the events of a twitch by admitting oxygen a few minutes after the twitch is over. In this manner four batches of heat are distinguished:

(1) Contraction heat.
(2) Relaxation heat.
(3) Delayed anaerobic heat.
(4) Oxidative heat.

(Groups (1) and (2) together are termed the "initial heat.") A complete knowledge of the chemistry of muscular contraction would permit us to describe the chemical events accompanying the twitch under these four heads. But it is doubtful if the events of groups (1) and (2) will ever be differentiated by chemical analysis. There is the difficulty that the changes accompanying a single twitch cannot be very extensive to judge from the minute heat accompaniment, and there is the even greater difficulty that it seems impossible to kill a muscle in the relaxed state. Only one experimenter has claimed to distinguish chemically between muscles in the contracted and muscles in the relaxed state. Embden observed some years ago that for a few seconds after a short tetanus muscles were insensitive to the momentary stimulation of immersion in liquid air, and he made use of this observation in the following manner (Embden and Jost, 1928). A muscle (A) was given a 5 sec. tetanus and plunged into liquid air 30 sec. later. It was thus killed in the relaxed condition. Its companion (B) was given only a single twitch and killed whilst contracted. Muscle B contained less hexosemonophate than muscle A. The experiment was, however, open to the objection that the immediate past history of the muscles was by no means identical. Other observers (Eggleton and Eggleton, 1929) are agreed as to the observation, but have interpreted it to mean that hexosephosphate accumulates during the tetanus. More recently these experiments have been repeated in a modified form which removes this ambiguity (Embden, Heffter and Lehnartz, 1930; and Embden and Jost, 1931), but the results are not so striking. Generally, though not always, the "contracted" muscle contains less lactacidogen than the "relaxed." No other attempts have been made to distinguish chemical changes during relaxation.

A few years ago a description of the changing state of a muscle performing a series of isometric twitches in nitrogen was a simple matter. The muscle produced heat and lactic acid and recorded a succession of tensions. It was found that as nearly as the accuracy of measurement permitted a relation existed between these three variables and a fourth, the length of the muscle; every kg. cm. of heat produced was accompanied by the production of 0·043 mg. of lactic acid and a total production of tension (adding together successive twitches) of 6·14 kg. per cm. length of the muscle. As far as could be seen, this triple relationship held true up to fairly advanced degrees of fatigue. Plotting the heat production at successive stages against the product ΣTl resulted in a straight line, and plotting lactic acid content gave the same straight line provided the ordinates were chosen so that 0·043 mg. of lactic acid were represented by the same length as each kg. cm. of heat (42 kg. cm.

of heat = 1 cal.). Increasing accuracy of measurement has revealed, as is often the case, that this simple relationship is only a first approximation. The two curves representing lactic acid and heat are no longer straight lines, and there is now a third curve to be considered, the inorganic phosphate production from phosphagen. (There are of course curves representing the changing ammonia content, lactacidogen content and doubtless changes as yet unknown, but the lactate and phosphate changes are by far the largest and most carefully studied of those known.)

Fig. 1, a comparatively simple chart, represents the probable course of an impossible experiment in which the phosphate and lactate production and heat production are measured continuously during a series of twitches leading ultimately to exhaustion. It has been constructed from the observations of several workers
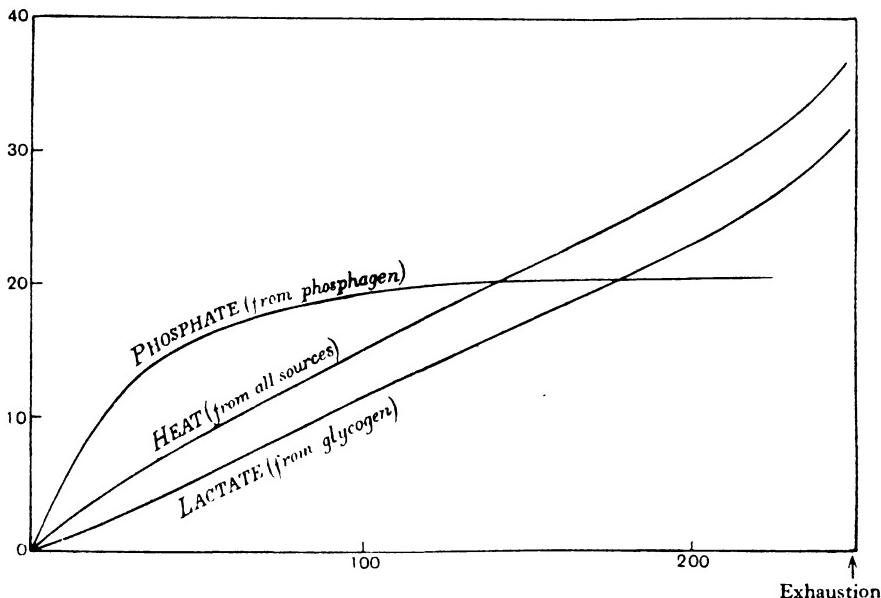
Fig. 1. Phosphate and lactate produced, and heat liberated by a sartorius muscle of a frog during a series of maximal twitches in nitrogen. Ordinates: kg. cm. of heat per gm., and micro-gm. mols per gm. (lactate and phosphate). Abscissæ: kg. cm. of tension-length. (For gastrocnemii double the values on the horizontal axis.) Some of the data from which this chart was assembled are to be found in references: Meyerhof and Schulz, 1931; Meyerhof and McCullagh and Schulz, 1930; Hill, 1931; Peters, 1913; and Hukuda, 1931.

published during the last five years. It will be observed that the muscle is represented as completely exhausted when it has liberated about a calorie of heat per gm. weight. This is the experience of several workers (Hukuda, 1931; and Peters, 1913). It will have produced about 35 micro gm. mols. of lactic acid (2·9 mg.) per gm. weight and on the average therefore it will have produced 2·9 mg. of lactate per calorie or about 350 cal. per gm. of lactic acid. Many of Meyerhof's earlier figures for this quotient were higher than this—the average is generally given as 385—but they referred to less severe fatigue.

Production of heat relative to total tension production is on the average 1 kg. cm. for every 6 kg. cm. of "tension-length," though in the very early stages and in advanced fatigue it is rather smaller than this. It is seen in fact that Tl/H starts out with a value of about 5 (Hill, 1931), but increases to such an extent that the mean value when the muscle is rather more than half fatigued is 6·7. It seems that the muscle requires a little exercise before it attains its best efficiency as a tension-producing device. The lessened efficiency in advanced fatigue was only to be expected. These figures refer to the sartorius muscle of the frog, but if we double the values of Tl along the horizontal scale, the chart becomes a truthful record of the gastrocnemius muscle, for, as Hill has recently shown (1931), the quotient Tl/H is twice as large for the gastrocnemius as for the sartorius. As Hill suggested, this is almost certainly due to the different distribution of the fibres in the gastrocnemius muscle. In passing it may be said that the value (deduced indirectly) previously in use was not 2 but about 1·45, and certain of the earlier results of Meyerhof and of Hill will need to be recalculated on this account.

The production of lactic acid by the steadily fatiguing muscle is relatively small in the earlier stages of fatigue. Indeed it is possible that none is produced in the first twitch or two. Taking an average for the first quarter of the curve, the chart indicates that the ratio

$$\frac{\text{Tension (kg.)} \times \text{Length (cm.)}}{\text{Lactate produced (mg.)}}$$

which Meyerhof has designated the "isometric coefficient for lactate" has the value 190 for gastrocnemii. This is in accordance with the recently published values of Meyerhof and Schulz (1931). As an average for greater degrees of fatigue it is found that this ratio drops to 150 and even to 115 in advanced fatigue.

The relationship of the lactic acid produced to the heat evolved is also seen from Fig. 1 to vary as the muscle becomes progressively fatigued. The heat produced per gm. of lactic acid (the "caloric quotient") should become progressively less according to the two curves in the figure, the value during the very early stages of fatigue being enormously high. There is no direct evidence for this enormously high initial value, but it is the necessary consequence of the facts that H/Tl is initially high (Hill, 1931) whilst lactate produced/Tl is initially low (Meyerhof and Schulz, 1931).

The progressive breakdown of phosphagen reflected by the rise in inorganic phosphate (for the phosphate does not arise from any other source) is most evident in the very early stages, and indeed practically ceases in the later stages. According to Meyerhof's results the ratio of the number of molecules of phosphate to the number of molecules of lactate produced in mild fatigue is about 3. Chemical technique does not permit evaluation of this ratio for the first two or three twitches: the changes are too small, but it is not unlikely that the ratio is very much higher at the beginning. Certainly it drops as longer and longer series are considered, and there is good evidence that the twitches of a fatigued muscle are not accompanied by any permanent increase in the orthophosphate content (see also Mackler, Olmsted and Simpson, 1929).

VI. FURTHER CONSIDERATION OF FATIGUE.

From the curves in the figure it is possible to make a deduction concerning the *p*H change of the muscle as it becomes progressively fatigued. The hydrolysis of phosphagen to creatine and phosphate is a reaction which leads to a liberation of base, for phosphoric acid is a weaker acid than creatinephosphoric, as was shown by Fiske and Subbarow (1929). The effect is greatest at a *p*H of about 5 and comparatively trivial at *p*H 8, but at *p*H 7 it is not negligible. Indeed it can be shown that if the production of a molecule of lactic acid from glycogen occurs simultaneously with the hydrolysis of three or more molecules of phosphagen, as is the case in the early stages of fatigue, the muscle should on this account become more alkaline. Later, when lactic acid production is not balanced by this compensating hydrolysis, the muscle should become progressively more acid. Such an effect has in fact been observed by Meyerhof, who followed the exchange of carbon dioxide between a muscle and the surrounding gas (a mixture of carbon dioxide and nitrogen) as it was progressively fatigued. At first the muscle took up carbon dioxide and as fatigue advanced gave it out again (Meyerhof and Lipmann, 1930).

The heat of hydrolysis of phosphagen has been measured by Meyerhof, and the heat of production of lactic acid from glycogen has been estimated, though necessarily with less directness, since the reaction cannot be brought about in pure solution. But given these figures and the curves in Fig. 1 it is obviously possible to see how far the actual heat produced by a muscle would be accounted for by the observed phosphate and lactate productions. Such calculations, however, though they havê been made (Hill and Parkinson, 1931), cannot be entirely satisfactory, for much depends upon the manner in which lactic acid is neutralised in the later stages of fatigue. Meyerhof is of the opinion that the necessary base is derived from protein: the heat of neutralisation of 1 gm. of lactic acid would in this case be about 100 cal. The heat of neutralisation of carbon dioxide allowed to diffuse into a resting muscle was found by Stella (1930) to be 9400 cal. per gm. mol. Meyerhof (1922) in experiments having the same object found 9900 cal. per gm. mol. for the neutralisation of valerianic acid in muscle. A similar value for the molecular heat in the case of lactic acid would mean the production of 100–110 cal. per gm.

There is still, moreover, the regrettable uncertainty as to the heat of combustion of glycogen. Slater (1924) made careful estimates of this quantity and found a value of 3836 cal. per gm. of dehydrated glycogen in dilute solution. Meyerhof (1922) has, however, found lower values, more in agreement with the early figures of Stohmann (1894). Slater's value leads to a calculated heat of formation of lactic acid in dilute solution, of 235 cal., Meyerhof's to about 170. About all that can be said is that the observed heat production seems to be accounted for more or less by the exothermic reactions discussed.

In the chart the muscle is represented as being completely exhausted when it has formed about 35 micro gm. mols. of lactate per gm. weight, that is to say when it contains about 300 mg. of lactate per 100 gm. A number of observers are agreed on this point. It is fairly certain that it is the accumulation of this lactate which

produces the exhaustion, for if the muscle be kept in Ringer's solution so that the lactate which is formed can diffuse away, fatigue does not become apparent so soon. Indeed it is now found that the limit is set by the amount of carbohydrate available. The amount of lactate which now accumulates in the Ringer's solution may easily exceed 1 per cent. of the muscle's weight. Hill and Kupalov (1929), who were the first to perform experiments of this type, further showed that if the Ringer's solution contained glucose even more energy could be liberated before fatigue set in. Indeed almost incredible activity is displayed by the frog's sartorius in these circumstances. A total of 10,000 twitches was performed in certain cases and the total tension produced was of the order of 6 tons per square cm. of cross-section.

This same technique has been used more recently by Gemmill (1932) to carry the argument a stage further. Taking muscles with different glycogen contents—the glycogen content being diminished in some cases by treating the frog with insulin—he fatigued all his muscles to exhaustion in Ringer's fluid sufficiently slowly to allow the lactate to diffuse away. Measurements of the total lactate produced and the total tension developed in the successive twitches showed that the amount of lactate produced in the muscle bore to the total tension-length exerted always the same relation (110 kg. cm. per mg. of lactate) although the actual amount of carbohydrate metabolised ranged from 2 to 12 mg. per gm. of muscle. This may be taken as a further indication that the ultimate source of energy in these circumstances is glycogen, though of course it leaves open the possibility of alternative sources for the muscle supplied with oxygen (see Ochoa, 1930).

VII. THE PRESENT WORKING HYPOTHESIS OF MUSCULAR MECHANISM.

Consideration of Fig. 1 raises yet another question. Are we to suppose that fresh muscles obtain their energy from phosphagen hydrolysis whilst fatigued muscles derive theirs from lactate production? One feels instinctively unwilling to adopt such a view and in fact the postulation of such a duplicate mechanism is unnecessary. There is now a considerable body of evidence to support the view first suggested by Lundsgaard (1930) that in all circumstances the energy of contraction is derived from the hydrolysis of phosphagen, and that in circumstances where there is no progressive diminution in the phosphagen content, the phosphagen breakdown associated with each twitch is being completely reversed by means of the energy released by lactic acid production, or if oxygen is available, some oxidative reaction. The amount of phosphagen which it is necessary to break down to provide the energy of a single twitch is probably less than 1 mg. per 100 gm. (assuming Meyerhof's value for the heat of hydrolysis). The skeletal muscle therefore carries an enormous reserve, and one is tempted to suppose that it is this fact which permits the high rate of energy production for limited periods possible to skeletal muscles.

Support for this theory of muscular contraction is provided by the fact that if a muscle is given a short maximal tetanus of, say, 2 sec. its phosphagen content can

be seen to rise again slightly during the next 20 sec., whilst simultaneously lactic acid is produced (Meyerhof and Schulz, 1931; and Meyerhof and Nachmansohn, 1930). The recovery is never complete in the cases which have been examined, but it is not unlikely that if muscles already fatigued, say two-thirds of the way to exhaustion, were examined, this "anaerobic restitution" would be found to be more marked. The production of lactic acid after the tetanus is over is a phenomenon repeatedly observed by Embden and his pupils for some years past (Embden and Lehnartz, 1928; and Lehnartz, 1931), though disputed until recently by Meyerhof (1928 *a*).

The theory accounts for the "delayed anaerobic heat" observed by Hill and Hartree (1920) to occur during the 3 or 4 min. after a tetanus or twitch. This heat is very small and rather variable (see Blaschko, 1930), and its origin has hitherto been unexplained. But if we suppose it to be the small balance between the exothermic delayed lactic acid production and the endothermic "anaerobic restitution" of phosphagen, the difficulty is met.

The theory provides an explanation for the comparatively small amount of phosphagen found in cardiac muscle. On the basis of Meyerhof's heat of hydrolysis the phosphagen reserve of the frog's heart would seem to be about sufficient for 10–20 beats. If the heart is deprived completely of oxygen the reserve vanishes, but the phosphagen content never sinks below a quantity sufficient for one or two beats (Clark, Eggleton and Eggleton, 1932). Since the heart cannot be tetanised there would be no necessity on the theory we are discussing for the large phosphagen reserve characteristic of skeletal muscle.

Finally, this view of muscular contraction survives without modification the strange behaviour of skeletal muscles poisoned with iodoacetic acid (I.A.A.). These muscles produce no lactic acid at all: they will go on contracting until they have no phosphagen left, and then die. The muscle is only capable of about a third of the energy output of the normal muscle. Hukuda (1931), for example, found that whereas the normal muscle gave out about 1 cal. per gm. in being fatigued to exhaustion anaerobically, the muscle poisoned with I.A.A. gave about 0·4 cal. (see also, Hill and Parkinson, 1931). The ratio Tl/H is found to have its usual value (Fischer, 1931): since lactic acid production is no longer contributing to the heat production, this fact suggests that phosphagen breakdown is likely to be enhanced. Such was indeed the experience of Meyerhof, Lundsgaard and Blaschko (1930), who found the heat production to be accounted for fairly satisfactorily by this enhanced phosphagen breakdown alone. Further, a most significant finding of Lundsgaard's (1930) is that the tension produced in a poisoned muscle is strictly proportional to the amount of phosphagen broken down, even up to exhaustion (51 kg. cm. per mg. phosphoric acid). We may picture then a normal muscle in the early stages of fatigue effecting a partial resynthesis of phosphagen between successive twitches, using glycolysis as a source of the necessary energy, whilst the poisoned muscle can effect not even a partial restitution. If oxygen is supplied the effect of I.A.A. is greatly lessened (Lundsgaard, 1930), indeed would probably vanish altogether if the oxygen supply were adequate. It seems that although the anaerobic muscle has no alternative to glycolysis as its ultimate source of energy

the aerobic muscle has alternative sources which are not rendered unavailable by I.A.A. This is shown very clearly by the experience of Clark, Eggleton and Eggleton (1932) with the frog's heart. This organ will go on beating aerobically for hours in the presence of a concentration of I.A.A. which in nitrogen would bring the heart to a standstill in twenty beats.

It seems from the work of Dudley (1931) and others (Barrenscheen, Braun and Dreguss, 1931) that I.A.A. inhibits the later stages in the glycolytic process, leaving untouched the conversion of glycogen into hexosephosphate and even into methylglyoxal, which represents probably the penultimate stage. Certainly glycogen disappears as usual and in some cases orthophosphate disappears also and hexosephosphate accumulates (for the nature of this hexosephosphate see Lundsgaard (1930). In the case of the heart muscle phosphate does not disappear (Clark, Eggleton and Eggleton, 1932) and in such a case we might expect to observe the accumulation of methyl glyoxal.

In this connection Vogt-Møller (1931) has shown that the effect of I.A.A. on the liver is to inhibit the methyl glyoxalase, and has produced good evidence that the symptoms of vitamin B_1 deficiency are due to the accumulation of methylglyoxal resulting from a similar inhibition.

VIII. THE ISOMETRIC TETANUS.

In the foregoing treatment of activity we have confined ourselves strictly to isometric contractions. The consideration of contractions in which work is performed raises some most interesting physical and mechanical problems, but is not known to present any peculiarities of a chemical nature, and does not therefore fall within the scope of this review. But a form of activity to which passing reference must be made is the isometric tetanus. At room temperature in the absence of oxygen an average frog sartorius maintaining maximal tension produces heat at approximately five hundred times its resting rate. It is of interest to compare this acceleration of metabolism with the value for the heart muscle of the frog, the metabolic rate (as indicated by oxygen consumption measurements) of which in full activity is only four or five times the resting metabolism (Clark, Stewart and Gaddie, 1930). The contrast is in part due to the considerably higher resting metabolic rate of the heart.

It is found, when allowance is made for the cost of initiating the tetanus, and for the heat evolution occurring at relaxation, that the maintenance of a steady tension by the sartorius of the frog is associated with the production of heat at a steady rate, which can be expressed in the form:

Heat produced per gm. of tension, per cm. length of muscle $= 4 \cdot 1 K \, gm.cm./sec.$

The value of K depends upon the temperature at which the experiment is performed and changes from a value of unity at $0°$ C. to one of about 10 at $20°$ C. K has in fact the value $3 \cdot 1^{0 \cdot 1 \theta}$, where θ is the temperature of the muscle (Feng, 1931). The interest of this work to the chemist lies in the fact that the chemical accompaniments of a tetanus change even more markedly with the degree of fatigue

of the muscle than is the case with a series of twitches, and contrast even more therefore with the constancy of the heat production. Thus Nachmansohn (1929) records that the phosphagen breakdown even in the third of a series of 2-sec. tetani is barely detectable[1]. Furthermore the phenomenon of after-production of lactate is much more in evidence when tetani are studied. It may be noted in passing that a temperature coefficient of 3·1 is too high for a physical process, and suggests that the controlling factor is a chemical reaction.

IX. OXIDATIVE RECOVERY IN ISOLATED MUSCLES.

We have considered so far activity in the presence and absence of oxygen. The discussion would not be complete without reference to the recovery in oxygen of muscles fatigued anaerobically. Here we are upon much less safe ground. Thermal measurements tell a simple story. The heat accompanying an isometric twitch, whether in oxygen or nitrogen, is the same. After relaxation is complete there follows in the anaerobic case a small and variable delayed anaerobic heat, the probable origin of which we have considered, and in the aerobic case a considerably larger heat evolution, just about equal in fact to the heat evolved during contraction and relaxation. We have to consider what chemical phenomena lie behind this aerobic recovery heat. A few years ago, before the existence of phosphagen was suspected, the explanation ran along these lines: the lactic acid, production of which during the twitch accounted exactly for the initial heat, was removed during the subsequent 3 or 4 min. in oxygen in two ways; about one-fifth was burned and the remaining four-fifths resynthesised to glycogen. This explanation was based upon the following facts: (1) neither the heat evolved nor the oxygen consumed was sufficient to account for the combustion of more than one-fifth of the lactic acid which disappeared; (2) the glycogen content of muscles thoroughly fatigued in nitrogen was said to increase during subsequent exposure to oxygen; (3) the respiratory quotient of the oxidative process in isolated muscles was found to be unity, indicating the combustion of either lactic acid or a carbohydrate, and the respiratory quotient of the extra metabolism of men after exercise was similarly found to be unity (Hill, Long and Lupton, 1924). These and other facts could be resumed in the form of a "balance sheet" of energy and material exchanges, with which the reader is doubtless familiar. This view of the matter is now exposed to very serious difficulties. (1) The lactic acid is partly and perhaps entirely produced after the muscle has relaxed (Meyerhof and Schulz, 1931). (2) Phosphagen is broken down during the twitch, and partly or completely reconstituted afterwards according as oxygen is withheld or supplied. (3) The evidence for the reformation of glycogen in the isolated muscle during oxidative recovery is not entirely satisfactory. (4) The "extra R.Q." of exercise in man is found to be unity only for a certain restricted range of conditions: after extremely rapid and violent exercise it may have values far above unity, even when allowance is made for the undue ex-

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[1] Since Nachmansohn killed his muscles 30 sec. after relaxation, thus permitting "anaerobic restitution" of phosphagen, this probably means that restitution after the third and subsequent tetani is complete in 30 sec.

pulsion and subsequent retention of carbon dioxide associated with the temporary acidaemia. Moreover, in very mild degrees of exercise there is often no evidence of any change in the respiratory quotient from the value characteristic of the individual at rest (Bock *et al.*, 1928).

Of these difficulties the third requires to be treated in greater detail. The evidence for the resynthesis of glycogen from lactic acid during oxidative recovery from fatigue in isolated frog muscles consists of six experiments published by Meyerhof in 1920. A satisfactory micro method for glycogen estimations was not at that time available. Meyerhof adopted the procedure of extracting the muscle three times with boiling water and estimating glycogen in the residue by the Pflüger method. The extraction with water necessarily removed some of the glycogen and in three of the experiments this error was reduced by determining the reducing sugar formed in a portion of the extract by acid hydrolysis. This, however, necessarily included carbohydrates other than glycogen. An increase was observed nevertheless in the amount of estimatable carbohydrate after oxidative recovery, of between 10 and 20 per cent. The only other evidence for the formation of carbohydrate from lactic acid by an isolated muscle was obtained later by Meyerhof, Lohmann and Meier (1925) who found an increase of from 5 to 15 per cent. in the total carbohydrate content of muscles immersed in Ringer's solution containing lactate. But perfusion of hind limbs with lactate Ringer led to no demonstrable carbohydrate synthesis (rise in four experiments, fall in three). True, the glycogen considered alone generally increased but apparently at the expense of other carbohydrates, not of lactate. It is a matter for surprise that muscles supplied with oxygen and lactate by perfusion should show no evidence of an ability to convert this lactate into carbohydrate, if an isolated muscle, receiving only such oxygen and lactate as can diffuse in from its surface, should have a demonstrable synthetic activity. In a repetition of the perfusion experiments Eggleton and Evans (1930 *a*) failed to record an increase (even in the glycogen content) which could be attributed to the utilisation of lactate, for the small and variable rise in glycogen content resulting from perfusion with lactate Ringer was equalled by a rise occurring with normal Ringer and therefore attributable in all probability to the conversion of some other form of carbohydrate into glycogen. Two valuable contributions to the technique of carbohydrate estimation in muscle have been made by Kerly (1930). Her experience emphasises the need for caution in attributing significance to small apparent changes.

The evidence for the ability of isolated muscles to convert lactic acid into glycogen is therefore not as satisfactory as it should be in view of the great theoretical importance attached to this ability. But even if we suspend judgment on this issue there remains the indubitable fact that the amount of lactic acid which disappears from a muscle during oxidative recovery cannot be accounted for even approximately by oxidation. The extra oxygen consumption and the extra carbon dioxide production will only account in fact for a quarter[1] of the lactate removal (Meyerhof 1930 *b*). The fact that the heat evolution is also inadequate for the hypothesis

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[1] The validity of considering only the extra respiration is not above question; but even if the total respiration during recovery is considered, the difficulty is not abolished.

of complete oxidative removal is not so serious a difficulty, for in the present state of knowledge we are free to postulate the existence of simultaneous endothermic reactions in order to cover the deficit. The possibility that some of the lactate which disappears is converted merely into methylglyoxal through the action of the glyoxalase of muscle is one which has never been sufficiently explored to permit an estimate of its probability (but see Lundsgaard, 1930).

X. THE VAPOUR PRESSURE OF ISOLATED MUSCLES.

Apart from the disadvantage to which reference has already been made, that it is impossible to kill a muscle in the relaxed condition, the technique of direct chemical analysis suffers from the drawback that continuous records of the events in a muscle are impossible. The muscle can only be killed once. It is their freedom from such drawbacks which gives physical methods of measurement much of their interest. It is possible to make a continuous series of practically instantaneous measurements of the thermal and mechanical accompaniments of muscle behaviour: measurements which, in themselves, add nothing to our knowledge of the chemistry of muscle, but which are of great value to the chemist in testing the validity of a hypothesis based simply upon chemical analysis.

To these two adjuncts, as the chemist regards them, a third is now added, namely the continuous recording of the osmotic pressure of the isolated muscle, or, strictly speaking, measurements of the vapour pressure. There is no need here to recount the details of the introduction and adaptation of this technique by Hill (1930). His apparatus has been described and with it he has shown that it is safe to regard the muscle from the point of view of vapour pressure as a solution in which all the water is "free," that is to say, available to dissolve the water-soluble constituents of the muscle. Since only 80 per cent. of the muscle is water, it is necessary always to bear in mind that if the concentration of a solute in the muscle— let us say urea or lactic acid—is 50 mg. per 100 gm., its osmotic pressure will be that of a solution of 50 mg. in 80 c.c., or 0·625 gm. per litre.

A resting sartorius muscle from a frog is found to have a vapour pressure equal to that of a 0·72 per cent. solution of NaCl, or, a notation preferable for some purposes, a concentration of 0·246 mols per litre of an ideal non-dissociating solute. The figure is not surprising in that empirical experience led long ago to the adoption of NaCl solutions of this strength as "isotonic" with frog tissue. It is not possible as yet to say with certainty to what extent the compounds known to be in muscle account for this total osmotic pressure, for it is only in the case of those constituents which can diffuse out of the muscle that an estimate can be made of their osmotic activity.

If a muscle is kept adequately supplied with oxygen its osmotic pressure remains unaltered. This is to be expected if the only reaction progressively occurring is the combustion of glycogen or other foodstuff stored in a colloidal and therefore osmotically negligible form. Kept in nitrogen, or better still, stimulated in nitrogen, the muscle increases its osmotic pressure, and the increase runs almost exactly parallel with the heat production up to quite advanced stages of fatigue. The

relationship can be expressed in the form that 8 cal. of heat are produced on the average along with every millimol of new osmotically active species. Knowing as we now do that the actual chemical accompaniments at different stages of fatigue change considerably, this constancy is very suggestive. The actual total rise in osmotic pressure when a muscle is fatigued to exhaustion in nitrogen is considerably larger than the known breakdown reactions added together would account for. It is equivalent to an increase from 0·72 per cent. NaCl to 1·07 per cent.—a 50 per cent. increase in the number of particles of all species dissolved in the muscle. To account for it in terms of lactic acid alone, for example, would require the production of about 9 mg. of lactic acid per gm. of muscle, at least three times the amount found by analysis. Throwing in the phosphagen breakdown known to occur, we can still only account for half of the observed change. If we suppose phosphagen to be contained in the muscle in combination with some colloidal constituent and therefore to exert a negligible osmotic pressure we can account for 75 per cent. of the observed change (Hill and Kupalov, 1930). The conclusion is forced upon us that unknown reactions of considerable magnitude occur in the stimulated muscle. If the muscle is allowed to go into rigor, there is a further rise in the osmotic pressure, which now becomes equal to that of a 1·17 per cent. NaCl solution.

The behaviour of anaerobic muscles treated with I.A.A. is of particular interest. Such muscles have a normal osmotic pressure whilst resting, but on stimulation to exhaustion the increase in osmotic pressure is only about two-fifths of that in a normal muscle (Hill and Parkinson, 1931). It will be remembered that the heat production is also about two-fifths of the normal. These facts in themselves, coupled with the observations that the total tension-length production is about two-fifths of the normal (Lundsgaard, 1930), that Tl/H for a single twitch has its normal value (Fischer, 1931), and that the distribution of heat between contraction, maintenance (in the case of tetanus) and relaxation is normal (Hartree, 1931; and Fischer, 1931) would suggest that the action of I.A.A. was not a qualitative one altering the nature of the chemical reactions associated with activity, but merely a quantitative one, reducing the muscle's "stamina." There is in fact no hint of that complete inhibition of both lactate and phosphate production revealed by chemical analysis. The effect of I.A.A. has been to suppress chemical and physical changes responsible for 0·6–0·7 cal. of heat per gm. of muscle, and for the production of an osmotic pressure equivalent to that of 0·2 per cent. NaCl. Very interesting, but at this stage rather speculative calculations can be made, on the basis of these observations, as to the nature of I.A.A. poisoning; but the interested reader should refer to the original papers. It must suffice here to say that Hill and Parkinson (1931) conclude that the unknown chemical reactions, suspected to occur in the normal muscle in view of the osmotic pressure findings, still occur in the poisoned muscle, and that the correlation of osmotic pressure rise with other measurements is rendered easier by the use of the hypothesis that the phosphagen of the muscle exists in a colloidal form and exerts therefore negligible osmotic pressure. It will be remembered that this possibility was raised several years ago by the observation

that phosphagen is unable to diffuse from living muscle although both creatine and phosphoric acid can, and creatinephosphoric acid is able to penetrate collodion (Eggleton and Eggleton, 1929).

The freezing-point measurements made by Meyerhof and Grollmann (1931) and by Meyerhof (1930 *a*) are on the whole in agreement with the vapour pressure measurements, though it must be admitted that the measurement of freezing-point is necessarily a less reliable indication of the state of a muscle at room temperature.

To the three biophysical criteria afforded by tension-length, heat, and vapour pressure measurements, it is to be hoped that a fourth may soon be added, which will be derived from measurements of electrical conductivity. A beginning was made in this direction several years ago by Hartree and Hill (1921) who ascertained that the conductivity of resting frog muscles (towards 90 cycle single phase A.C.) was equal to that of a 0·36 per cent. NaCl solution at all temperatures between 0° and 20°. These experiments had only a utilitarian purpose in connection with the calibration of thermopiles and were not carried any further. More recently conductivity measurements have been made by Landsborough Thomson (1928), who has thereby been able to distinguish between normal and inexcitable muscles. This author used A.C. of very high frequency —10^7 cycles—and his values for conductivity were only one-half as great as those of Hartree and Hill. Of particular value would be comparisons between muscles in the contracted, and muscles in the relaxed condition. The electrical conductivity of a living tissue is doubtless by no means so simple a phenomenon as the conductivity of a salt solution and the utmost caution would have to be used in interpreting the measurements.

XI. CATABOLIC CHANGES IN CELL-FREE MUSCLE EXTRACTS.

Of the chemical processes accompanying muscular fatigue those connected with carbohydrate metabolism have in the past received most attention, and the anaerobic processes leading to the production of lactic acid have in particular been worked out in great detail by study of certain of the enzymic properties of aqueous extracts of muscle. Serious work on this subject probably dates from the publication by Embden, Kalberlagh and Engel (1912) of some experiments in which it was observed that muscle juice failed to convert glycogen into lactic acid, and that the spontaneous formation of lactic acid in such juices was accompanied by the production of approximately equivalent amounts of phosphate. Starting from this observation a considerable body of evidence has been built up by this school emphasising the importance of hexosephosphoric esters in muscular contraction. In particular much valuable information was gained by Laquer, one of Embden's pupils (1921, 1922 and 1923). The same class of phenomena has been the subject of close attention by Meyerhof and his collaborators whose interest was, however, centred rather upon the enzyme system responsible for the production of lactic acid. A considerable technical advance was made by Meyerhof (1926) in the introduction of methods of preparing cell-free muscle extracts which were incidentally nearly carbohydrate-free, and could be regarded as enzyme preparations whose influence on any desired substrate could be ascertained without complication.

The condition of the subject in 1928 was reviewed by the writer elsewhere and need not be here repeated. In the last few years this process of isolating the fermentative system has been carried further, and certain components of the system have been isolated and identified. The system consists of an enzyme and co-enzyme, though the term co-enzyme has proved to be rather misleadingly simple. It had early been found that inorganic phosphate was a necessary constituent of the system, and that hexosephosphates were formed intermediately in the transformation of glycogen to lactic acid. It is now known that in addition magnesium ions are essential, and adenylic acid. This new departure originates from the discovery by Lehnartz (1928) that the addition of adenylic acid to the juice of a rabbit muscle containing 2 per cent. of bicarbonate causes a very rapid disappearance of inorganic phosphate. The optimum reaction for this effect proved to be pH 7·3 and it was found that practically all the inorganic phosphate had disappeared in a minute at room temperature. The effect was due to the formation of adenylpyrophosphoric acid (adenyltriphosphoric acid). This synthesis began slowly to be reversed after a few minutes and the compound broke up. But at this stage phosphagen appeared and increased in amount, particularly if the reaction was made alkaline. Incubation of the muscle extracts in bicarbonate without any addition brought about a synthesis of phosphagen (optimum at pH 8) reaching a maximum in about two hours. This synthesis also occurred, though with an initial delay, in the presence of added adenylic acid. Both of these syntheses require energy, but at this stage it was not possible to suggest a source of this energy.

Meyerhof and Lohmann, studying the hydrolysis of phosphagen in muscle extracts, showed that it produced about 115 cal. per gm. of phosphoric acid liberated, and showed further that the enzyme responsible can be inactivated by allowing the extract to stand some hours at room temperature. The optimum reaction for the breakdown was between pH 6·4 and 7. At pH 8·5 the enzyme was inert, whilst in more alkaline reactions (9·5–10) synthesis of phosphagen was observed provided glycogen or starch was present. This suggested that lactic acid formation might provide the necessary energy for synthesis, but it was found that the lactic acid production was too small. Meyerhof and Lohmann have suggested recently that the energy required for this alkaline synthesis of phosphagen is derived from the breakdown of adenylpyrophosphoric acid into inosinic acid, phosphate and ammonia. The breakdown reaction yields 170 cal. per gm. phosphoric acid, whilst the synthesis only requires 120 cal. In support of this they adduce the fact that if the muscle extract has already lost its adenylpyrophosphoric acid by autolysis, addition of alkali no longer promotes phosphagen synthesis. Addition of adenylic acid is of no assistance, but the triphosphate produces an effect in proportion to the amount added. If glycogen is added as well a greater phosphagen synthesis is possible. Thus the ultimate source of energy would appear to be the breakdown of glycogen to lactic acid. The energy produced by this reaction promotes the synthesis of adenylpyrophosphoric acid which in turn energises the phosphagen synthesis by its own breakdown. The adenylic acid therefore is to be pictured as going round and round a cycle taking energy from glycogen and using it for the

formation of phosphagen. It is to be presumed that Lehnartz's muscle extracts contained sufficient glycogen or other carbohydrate to finance the synthesis of adenylpyrophosphoric acid.

The cell-free extract of muscle is incapable of respiration. The sum total of the reactions going on in it must of necessity liberate energy. The chief of these, and the one most studied is the liberation of lactic acid from glycogen. It seems that we have to consider a system of which the known constituents are all those mentioned above together with magnesium ions and a number of enzymes; and the exact nature of the events occurring is modified considerably by the absence of certain of these, and by such considerations as the temperature, age and reaction of the extract. For example, the extract must contain magnesium in order to convert glycogen, hexosemono-, or hexosediphosphate into lactic acid, though the concentration of magnesium required is considerably less the more phosphorylated is the substrate. Similarly the extract hydrolyses phosphagen if the reaction is less alkaline than pH 8, but in a more alkaline reaction a synthesis will occur provided adenylpyrophosphoric acid is present, which by its breakdown to inosinic acid, ammonia and phosphate appears to be able to provide the necessary energy. This breakdown can itself be reversed with the aid of the energy derived by the conversion of glycogen to lactic acid. Thus Lehnartz's original observation that addition of adenylic acid to a muscle extract caused a very rapid disappearance of inorganic phosphate was due to the formation of adenylpyrophosphoric acid at the expense of lactic acid formation and the subsequent synthesis of phosphagen was coupled with a breakdown of adenylpyrophosphoric acid.

XII. EXERCISE IN THE INTACT ANIMAL.

Very little can be achieved in the study of isolated mammalian muscles owing mainly to difficulties of oxygen supply. Analyses of resting muscles, removed and killed with the utmost rapidity, have been made, with results quantitatively very similar to those of resting frog muscles (Sacks and Davenport, 1928). The effects of oxygen lack can be studied (all too easily!) and are found to be entirely similar to the case of the frog. Phosphagen disappears very rapidly (Irving and Bastedo, 1928) and lactic acid accumulates until a concentration of about 0·6 per cent. is reached in rigor (Smith, 1930). Myothermic measurements made by E. Fischer (1930) on the isolated rectus muscle of the mouse at $37°$ gave results entirely similar to those obtained with frog muscle at $20°$.

But if the mammal is an unsuitable source for isolated muscles, it lends itself well to a different line of work—the study of the blood in relation to exercise. Generally speaking, 2–5 c.c. of blood are needed for the estimation of two or three substances, and for an experiment involving 6–12 samples over a period of an hour or two, the blood required is 12–60 c.c. If this is to be a negligible fraction of the animal's blood, the animal must have, say, 120–600 c.c. of blood, that is the animal must weigh 2–9 kg. at least.

The study of exercise in intact animals is not, and from the nature of things,

may never be, sufficiently complete to reveal much concerning the chemistry of muscular contraction. Such knowledge as is now at our disposal in no way conflicts with the view of muscular contraction put forward in an earlier section of this article: on the other hand it does not help us very much further, save in the one very important respect that it gives some assurance that the events occurring in the muscle are not seriously altered by isolation from the body. Mild degrees of exercise in intact mammals may lead to a slight raising of the respiratory quotient, but quite often do not. It seems that, provided no serious oxygen debt is incurred, the animal continues to burn the three primary foodstuffs in unaltered proportions. In more severe fatigue the ratio of the extra carbon dioxide eliminated to the extra oxygen required is often unity (Hill, Long and Lupton, 1924). In exercise of this severity lactic acid accumulates temporarily in the blood. This is in accordance with the view that the production of lactate from carbohydrate is the only source of energy for muscle fibres lacking oxygen and the extra respiratory quotient of unity suggests that in accordance with a sort of mass action law the body is burning this lactate at the earliest opportunity. In extremely severe exercise, such as produces a serious acidosis, this extra respiratory quotient may have values exceeding unity (Best, Furusawa and Ridout, 1929). This puzzling feature has not yet been explained, though it may be associated with the utilisation of calcium carbonate reserve in the bones (Ferguson, Irving and Plewes, 1929; Irving, Ferguson and Plewes, 1930; and Kilborn, 1928) if we suppose that the restitution of this calcium carbonate is a process so slow and gradual that respiratory measurements are unable to detect it. There occurs during exercise an increase in the creatine (Kácl, 1932) and phosphate (Havard and Reay, 1926; and Owles, 1930) concentration of the blood, though this is never so striking as the rise in the amount of lactate. But the question of most interest which we can ask of the experimenter with intact animals is, what exactly becomes of the lactate when it disappears again. Living cells are capable of remarkable chemical feats, but the production of glycogen from lactic acid is a feat of which we require very convincing evidence. There is no doubt that the glycogen which disappears from the muscles of a more or less intact mammal reappears again, but there seems to be no correlation either in time or stoichiometrically between the three phenomena of glycogen restitution, lactate removal and extra oxygen consumption. For example it is recorded by Martin, Field and Hall (1929) that no quantitative relation holds between the lactate disappearance from the body after exercise and the excess oxygen consumption during the same period; and Gollwitzer, Meier and Simmonson (1929) report that after moderate exercise the oxygen consumption rate returns to normal before the blood lactate concentration. In the first few minutes after exercise, according to these authors, the oxygen consumption more than accounts for the lactate which is disappearing while the reverse is the case towards the end of the recovery period. Long and Grant (1930) report a similar discrepancy between lactate removal and glycogen restitution in the bodies of rats after exercise. Although the temporary lactate accumulation had been dispersed in two hours, the glycogen content of their bodies was not back to normal even in five hours. See also Himwich and Rose (1929), Jahn (1930) and

Hattingberg (1929); and also Stewart, Gaddie and Dunlop (1931), and Rapport (1929).

That glycogen reappears in the muscles of an intact animal or decerebrate preparation during oxidative recovery from fatigue is undeniable. There is evidence that it will even occur after removal of the pancreas and intestines and ligature of the hepatic artery and portal vein (Cleghorn and Peterson, 1932), although certain physiologists (*e.g.* Debois, 1930; and Hoet and Ernaud, 1931) are of the view that removal of the pancreas or even denervation of the pancreas prevents this resynthesis. But it does not follow that muscles are converting lactic acid into glycogen. The glycogen may be derived in such cases from other carbohydrate present in the muscle (of which there is often a considerable amount) or even from the blood sugar, which latter could be replenished by the hydrolysis of liver glycogen. If it be objected that in the eviscerate preparation the blood sugar does not fall during the period of glycogen synthesis sufficiently to account for the glycogen formed, it can be argued that the liver is in these preparations left attached to the circulation by the hepatic veins. With its oxygen supply stopped by ligation of the arteries, the liver is likely to form glucose from glycogen all the more readily, and sufficient of this sugar may be carried by the ebb and flow in the hepatic veins into the circulation. The respiratory quotient of such preparations has frequently been found to be unity, and the disappearance of lactate might be accounted for by oxidation alone. Eggleton and Evans (1930 *b*) using a pump-lung-hind-limb preparation (dog) have demonstrated the removal of lactate by the muscles from the blood in such preparations, but unfortunately they did not determine the total carbohydrate content of the muscles. Such an experiment is necessary to test finally the ability of mammalian muscles to synthesise carbohydrate of any kind from lactate.

The problem of lactate removal in intact mammals is complicated by the interfering action of anaesthetics. It has been known for many years that ether induces a considerable rise in the blood sugar concentration, and Evans, Tsai and Young (1931) have recently succeeded in demonstrating that this is due to an intense glucogenesis brought about in the liver. Even ether-induced anaesthesia followed by decerebration or decapitation results in a temporary discharge of 60 per cent. of the liver glycogen, followed by a slow recovery lasting several hours. Such preparations clearly provide no safe base line for experiments designed to study the liver in relation to carbohydrate metabolism. (The glycogen content of the muscles was found to suffer no appreciable change.) It has been thought, since it was found by Deuel and Chambers (1924) that the blood sugar level was not affected by the use of amytal, that this anaesthetic is safe for such purposes. But Evans and his collaborators (1931) could find no steady state as regards the liver glycogen in animals anaesthetised with this drug. The glycogen content of the liver fell slowly but continuously for some hours.

XIII. INVERTEBRATE MUSCLE.

It was noticed in 1928 by the Eggletons that phosphagen was not present in the muscles of such invertebrates as they examined, although present in all the vertebrates tested and even in the primitive chordate amphioxus. They observed that here appeared to be a biochemical distinction between the two great phyla of animals, and speculated on the possibilities of "chemical mutation" in the science of genetics. The distinction was in fact not altogether new. Kutscher (1914) had observed years before that creatine was absent from invertebrates and that "arginine takes its place." Meyerhof showed (1928 c) that invertebrate muscles contain argininephosphoric acid, apparently in the place of creatinephosphoric acid. The former has similar properties to the latter, but its hydrolysis by acid is inhibited in the presence of molybdate, whereas in the latter case it is greatly accelerated. This fact forms the basis of one method of distinguishing the two substituted guanidinephosphoric acids. Not a great deal of work has so far been accomplished on the physiological function of argininephosphoric acid in invertebrate muscle, but the results so far published (Needham et al., 1932 a; and Meyerhof, 1928 b) indicate that it serves an entirely similar purpose to that of creatinephosphoric acid. Thus it disappears from a muscle in fatigue, and reappears during oxidative recovery, the changes being compensated by changes in the free arginine and phosphate content.

In the original planning of this review a detailed discussion of the problems of phylogeny raised by the distribution of these two substances in the animal world was projected, but this aspect of muscle chemistry has recently received attention in a review by J. Needham (1932) and is treated in the present number of *Biological Reviews* by E. Baldwin. To these articles the reader is referred. Needham and his colleagues have established the highly significant fact that in the muscles of certain animals both the phosphagens, creatinephosphoric acid and argininephosphoric acid, are present. The possibility is thus reopened of a gradual evolutionary transition from the use of the one compound to the use of the other (Needham et al., 1932 a and b.)

XIV. SUMMARY.

1. In the last few years it has been found that:

(a) A large proportion of the acid-soluble phosphate originally classed as "inorganic" is actually combined with creatine (creatinephosphoric acid, phosphocreatine, phosphagen).

(b) Most of the organic phosphate hydrolysed by the muscle enzymes during autolysis (the fraction hitherto known as "lactacidogen") is adenosinetriphosphoric acid (adenylpyrophosphate).

(c) There is no hexosediphosphoric ester in normal muscles, but a small amount of a hexosemonophosphoric ester. To this the name lactacidogen is now applied.

2. Concerning the chemical events accompanying activity of a muscle, two discoveries in particular have greatly altered their interpretation:

(a) It is found that the production of lactic acid occurs partly, and in some cases entirely, after the activity has ceased.

(*b*) The production of lactic acid does not occur at all in muscles suitably poisoned with fluoride or iodoacetate. In such muscles the energy production is proportional to the extent of the accompanying phosphagen breakdown and is limited by the amount of phosphagen in reserve.

3. Arising out of these and other considerations discussed in the text, a reasonable working hypothesis of muscular contraction is that the thermal and mechanical energy released in activity comes from the (exothermic) breakdown of phosphagen. During the subsequent recovery period this phosphagen is resynthesised, the necessary energy being derived:

(*a*) In the absence of oxygen; from the conversion of glycogen to lactic acid. Restitution in this case is only complete in muscles which have used up nearly all their reserve of phosphagen. There seems to be no alternative to the glycolysis mechanism as an anaerobic source of energy.

(*b*) In the presence of oxygen, from the combustion probably of any available foodstuff, though carbohydrate seems to be the material used for choice by skeletal muscles.

4. Other directions in which rapid advance has been made in recent years are:

(*a*) The isolation of the constituent parts of the ferment system responsible for the glycolytic process in muscle.

(*b*) The perfection of a delicate and rapid method of measuring the vapour pressure of muscles. One of the immediate results of the application of this technique has been the demonstration that the catabolic changes so far studied in connection with muscular activity do not account for all the osmotic pressure change. There must remain reactions of quite considerable extent so far undiscovered.

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PHOSPHAGEN

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(With One Text-figure.)

CONTENTS.

I. INTRODUCTORY AND GENERAL.

Now that phosphagen has become a household name among biochemists and physiologists it seems a little surprising that it was not discovered before. As long ago as 1832 Chevreul described to the French Academy of Sciences a new organic substance, obtained from meat extract, to which he gave the name creatine. He did not analyse his product, and in fact did no more than hazard a false conjecture as to its composition, and during the next few years his discovery was in the way of being forgotten, for a number of workers tried without success to confirm it. But Liebig, in 1847, was able to show that, by a suitable method, creatine might be separated from the flesh of a number of mammals, birds, and fishes that he examined. By 1853 the presence of creatine had been demonstrated in the muscles of all classes of vertebrates.

The relation of creatine to muscular activity has often enough been discussed, but until the work of Eggleton & Eggleton and of Fiske & Subbarow, in 1927, no idea was obtained as to the real nature of this relation. Comparison of the creatine content of various muscles with their activity revealed a marked parallel, while the smooth muscles of the gut and uterus showed a significantly low creatine concentration. On p. 157 of his monograph on creatine and creatinine, Hunter (1928) remarks: "as a historical curiosity it is perhaps worth mentioning that Valenciennes

& Frémy (1855) claimed to have frequently found in muscles *creatinine* in combination with phosphoric acid. Anticipatory hints of greater value are perhaps to be found in the observation of Riesser (1922) that the creatine content of different muscles is closely paralleled by their content of lactacidogen, and in the belief expressed by V. C. Myers (1922) that 'glycogen, creatine, phosphoric acid, and potassium are closely associated in active muscle'."

The relation between creatine and muscular activity has been chiefly discussed with reference to the output of creatinine. Folin (1905), using his newly devised method for the estimation of creatinine, found that the output of creatinine is, upon a meat-free diet, "a constant quantity, differing for different individuals, but wholly independent of quantitative changes in the total amount of nitrogen eliminated." A number of workers spent a great deal of time in showing that the constancy of the daily output of creatinine is not so marked as Folin's dictum would appear to have it, but in the main, Folin's statement still stands when the output is taken over any length of time. Pekelharing (1911) and Schultze (1921) investigated the effects of prolonged tonic contraction and of exercise upon the creatinine excretion, and although there was no appreciable change in the total *daily* output of creatinine in their experiments, it is quite evident that, during the periods in which the activity took place, there was a slight increase in the amount of creatinine eliminated. These workers regarded the creatinine output as an index of the muscle tone, and of the activity in the respective cases, while Myers & Fine (1913) regarded it as a direct index of the muscle creatine itself.

As for the site of formation of creatine, this is not the place to enter into a discussion of the various theories that have been put forward from time to time, nor can we here stop to consider the many problems in connection with the intermediary metabolism of creatine and creatinine. Enough has been said to indicate the wealth of pre-existing evidence for a close relationship between creatine and muscular activity. It is to be hoped that the discovery of the compound of creatine with phosphoric acid, to which the name of phosphagen has been given, will help to clear up the at present rather chaotic state of our knowledge of the intermediary metabolism of creatine itself.

This discovery has done more than to reveal at last, for example, the nature of the creatine complex postulated by Folin & Denis (1914) and others to explain a number of otherwise inexplicable facts. At the time of the discovery, the Hill-Meyerhof theory of muscular contraction reigned supreme, standing as it did for a vast amount of brilliant work and careful thought. None the less, there were gaps in the scheme brought forward by the workers of this school. Presently the work of Lundsgaard (1930 *a* and later papers) appeared, and drastic revision of former views became necessary. The Hill-Meyerhof theory regarded the breakdown of glycogen to lactic acid as the more or less direct energy source of the muscle. Yet, by poisoning muscles with iodoacetic acid, Lundsgaard was able to show that muscular contraction can take place without any lactic acid production whatever, although phosphagen is broken down as in a normal muscle.

This, indeed, is not so very surprising now that we have grown accustomed to the

idea. It seems highly probable that the lactic acid mechanism is a property common to all animal cells, and that it perhaps represents the fundamental source of energy in animal organisms. If this is so, one would expect that the amount of lactic acid produced by any given cell would be proportional to the energy expenditure to which the cell is put, and since the muscles do the major part of the work of the entire body, it is to be expected that they will show a relatively enormous production of lactic acid. Looking at the matter from this point of view, it is difficult to see why the muscle should be expected to derive its energy *directly* from the breakdown of carbohydrate. The muscle, being, as it is, a highly specialised structure, would surely be expected to have some correspondingly highly specialised system for dealing with the problem of converting its chemical into mechanical energy. In any case, the production of lactic acid is too slow a process to be able to release, at a moment's notice, energy at such a rate as it is needed in the contracting muscle. The presence of some more rapidly acting system than the lactic acid mechanism would be thought to be essential. If one may speak in terms of power, it is legitimate to say that the lactic acid mechanism cannot, or at any rate does not, work at a sufficiently high rate to bring about the rapid liberation of energy that one sees, for example, in the gastrocnemius of the frog. As the situation now appears, one may regard the lactic acid mechanism as a relatively slow process used by the muscle to renew its store of readily available energy, much as a lever is used to reset the spring of an air-gun.

We cannot suppose that the phosphagen breakdown is brought about directly by the application of a stimulus to the nerve or to the muscle itself, for Dulière & Horton (1929) have shown that a muscle may, after suitable treatment, pass into a state of (reversible) inexcitability in which a great number of stimuli can be applied to it without causing either a contraction or any breakdown of phosphagen. It seems likely that ionic permeabilities and surface potentials must play a part in determining the transmission of the stimulus to the chemical mechanisms that we know, but we have little or no definite knowledge as to how this is brought about. Before the energy significance of phosphagen breakdown was realised, it was suggested that phosphagen might be concerned with the excitation process (Nachmansohn, 1928, 1929). More recent work, however, makes this seem improbable. Again, we cannot at present regard the hydrolysis of phosphagen as the direct source of the contraction energy. The colloidal cell constituents probably play a great part here. In some way the chemical energy of the phosphagen breakdown must be brought to bear upon the actual mechanical structures of the cell, causing them to shorten. The way in which this is brought about is a matter for a very great deal of future research, and we cannot claim to have more than a fragmentary knowledge of the actual working of the cell until we learn how the conversion of chemical to mechanical energy is brought about within it. But the discovery of phosphagen in nerve by Gerard & Wallen (1929) suggests that phosphagen may be concerned also in the transmission of the nerve impulse. It can scarcely be coincidence that muscle, nerve, and electrical organs, all of which contain phosphagen, count electrical changes among the manifestations of their activity, suggesting that

these changes may perhaps be of far greater significance than their magnitudes, in the first two cases at any rate, appear to indicate.

II. (a) CREATINE AND PHOSPHAGEN IN PHYLOGENY.

Long before the existence of creatine phosphate in muscle was ever suspected, the presence of creatine itself had been satisfactorily demonstrated in members of every class of the vertebrate phylum. An admirable discussion of the biological distribution of creatine and creatinine is given by Hunter (1928). Such primitive forms as *Amphioxus, Petromyzon,* and the larval form of the latter (*Ammocoetes*), were found to contain creatine in their muscles. In *Acanthias*, one of the lowest of the fishes, and in the still more primitive *Petromyzon*, creatine was found to be associated with the related substance, betaine. On account of the way in which creatine is mainly confined to muscular tissues, it is interesting to notice that the presence of creatine has been demonstrated in the electrical organ of *Torpedo*, the electric ray, in which the organ in question is believed to be derived from functionally modified muscle cells. The creatine content of muscles in general seems to be closely related to their activity. For example, white muscle is richer than red, cardiac muscle contains less than either of these, while the smooth muscles of the gut and uterus contain only very little. Non-muscular tissues, *e.g.* the blood, brain, etc., contain little or none.

The question of the presence or absence of creatine in or from invertebrate tissues has long been a matter of contention. Delaunay (1931) gives a number of references to various authors who claim to have detected it in the bloods of echinoderms and molluscs, while Hunter also gives a critical discussion of the evidence. It is sufficient for the present purposes to say that until recently the evidence either way has never been very convincing. Such evidence as there was for the presence of creatine and creatinine in invertebrate tissues was usually based upon the notoriously unspecific Jaffé reaction; Hunter (1928, p. 110), after a long list of so-called demonstrations of its presence, remarks: "against positive findings so imperfectly supported there are to be set a long array of failures to detect, by isolation or by characteristic reactions, either creatine or creatinine in invertebrate material." On the other hand, however, the muscles of the crab, lobster and crayfish have long been known to abound in arginine, which, like creatine, is a guanidine derivative. Why these two substances should be interchanged is a matter of conjecture, but it is interesting to note in this connection that while the enzyme arginase[1], which hydrolyses arginine to give urea and ornithine, is widely distributed in vertebrate tissues, it appears to be totally lacking in invertebrates (see Hunter & Dauphinee, 1924). Such a sharp change-over is postulated by Kutscher (1914) who believes that arginine is never to be found in the free state in vertebrate tissues, and cannot confirm (1931) the recent finding of small amounts of free arginine in vertebrate skeletal muscle claimed by Kiech, Luck & Smith (1931).

[1] Our knowledge of the function of this enzyme has recently been greatly extended by the researches of Krebs & Henseleit (1932) and it seems likely that some explanation of the facts here mentioned may soon be forthcoming.

On account of the close relation between the creatine content of a muscle and its activity, it has been supposed by many authors that creatine plays some important part in muscular activity, though the nature of its importance has never been appreciated till recently.

The two forms of phosphagen at present known, viz. creatine- and arginine-phosphoric acids, seem to follow the same general line of distribution already noticed in the case of their basic components. In general, C-P is found alone in vertebrate tissues, even so far down in the scale as *Amphioxus* (Eggleton & Eggleton, 1928; Meyerhof, 1928), while A-P is found alone in the muscles of invertebrates. The Eggletons (1928) have looked in vain for C-P in a number of invertebrates, and are confirmed by Meyerhof (1928), and by Needham, Needham, Baldwin & Yudkin (1932), while on the other hand, Needham & Baldwin (1931) have been unable to detect A-P in the stomach or cardiac muscle of the frog and rat. Finally there is, as yet at any rate, no evidence for the occurrence of A-P in vertebrate skeletal muscles. In two cases, however, the two phosphagens have been found co-existing, viz. in the jaw muscles of *Strongylocentrotus lividus*, and in the body of the entero-pneust, *Balanoglossus salmoneus* (Needham, Needham, Baldwin & Yudkin, 1932). According to the most generally accepted theory of the ancestry of vertebrates, namely that of Bateson, MacBride & Garstang, the echinoderms and the entero-pneusts are to be considered as possessing the closest affinities to the vertebrates, and it is very interesting to find that the morphological evidence upon which this theory rests receives support from so unexpected a quarter. The new chemical evidence for the relationships in question, and the phylogenetic significance of the observations in question have been fully discussed elsewhere by Needham, Needham, Baldwin & Yudkin (1932) and by Needham & Needham (1932).

Like creatine itself (Beker, 1913), creatine phosphagen is found in greater amounts in skeletal than in cardiac muscle, and here again in greater amounts than in gut muscle (Eggleton & Eggleton, 1929), and is evidently intimately associated with muscular activity, its precise rôle in which will be discussed presently. In view of the large difference of concentration between the phosphagen of skeletal and that of gut muscles, it might be thought that creatine phosphagen is a feature of striated, and arginine phosphagen a feature of unstriated muscle. This is not the case. The heart and stomach muscles of the rat and frog contain no arginine phosphate (Needham & Baldwin, 1931), the striped jaw muscles of *Strongylocentrotus lividus* contain both (Needham, Needham, Baldwin & Yudkin, 1932), while both the striped and the plain adductors of *Pecten* contain only arginine phosphate (Meyerhof, 1928).

Arginine phosphate has been detected in planarian worms in which the motion is largely, though not entirely, brought about by ciliary activity, and as it has also been detected in the ctenophore, *Pleurobrachia pileus*, which contains no true muscle, this seems to support the suggestion that perhaps both ciliary and muscular activity derive their energy from similar chemical processes. Again, arginine phosphate was detected in considerable amounts in the swimming plutei and gastrulae of *Strongy-locentrotus lividus* (Needham, Needham, Baldwin & Yudkin, 1932), a circumstance which lends still more support to this suggestion, since the locomotion here is

TABLE I. Taken from Needham & Needham (1932).

Group	Genus and species	Portion used	Total P	Mg. P per gm. wet wt. — Inorg. P	Mg. P per gm. wet wt. — Creat. P	Mg. P per gm. wet wt. — Arg. P	Phosphagen P in % of total P — Creatine	Phosphagen P in % of total P — Arginine	Phosphagen P in % of total P — Total	Authors
Coelenterata	*Aurelia* (sp.?)	Contractile tissue at circumference	—	0·007	0·000	—	0·00	—	0·0	EE
	Anthea rustica	Body wall	0·030	0·031	0·000	0·000	0·00	0·00	0·0	NNBY
Platyhelminthes	*Pleurobrachia pileus*	Whole body	0·011	0·0065	0·000	0·0048	0·00	42·0	42·0	NNBY
	Planaria vitta	"	0·141	0·106	0·000	0·035	0·00	24·8	24·8	NNBY
Nemertinea	*Polycelis nigra*	"	0·182	0·155	0·000	0·027	0·00	14·8	14·8	NNBY
	Lineus longissimus	"	0·467	0·223	0·000	0·244	0·00	52·5	52·5	NNBY
Annelida	*Lumbricus* (sp.?)	"	—	trace	0·000	—	0·00	—	—	EE
	Sabellaria alveolata	"	0·321	0·224	0·000	0·097	0·00	30·2	30·2	NNBY
	Spirographis brevispira	"	0·271	0·099	0·000	0·172	0·00	63·5	63·5	NNBY
	Nereis diversicolor	"	0·352	0·215	0·000	0·137	0·00	37·0	37·0	NNBY
Podaxonia	*Sipunculus nudus*	Retractor muscles	0·550	0·210	—	0·340	0·00	62·0	—	M
	Sipunculus nudus	Body wall	0·452	0·133	0·000	0·319	0·00	71·0	71·0	NNBY
Mollusca	*Aplysia* (sp.?)	Foot muscles	—	0·020	0·000	—	0·00	—	—	EE
	Pecten (sp.?)	Adductor muscle	—	1·140	0·000	—	0·00	—	—	EE
	Pecten opercularis	Striped adductor muscle	0·640	0·250	—	0·380	—	60·0	—	M
	Pecten jacobaeus	" "	0·710	0·250	—	0·460	—	66·0	—	M
	Mytilus (sp.?)	" "	—	0·500	0·000	—	0·00	—	—	EE
	Sepia officinalis	Mantle muscle	1·750	1·520	0·000	0·230	0·00	13·2	13·2	NNBY
	Octopus vulgaris	" "	1·340	0·888	0·000	0·452	0·00	33·5	33·5	NNBY
Echinodermata	*Holothuria* (sp.?)	Longitudinal muscles	—	0·120	0·000	—	0·00	—	—	EE
	Holothuria tubulosa	" "	0·320	0·050	—	0·280	—	86·0	—	M
	Stichopus (sp.?)	" "	0·380	0·140	—	0·250	—	65·0	—	M
	Synapta inhaerans	" "	0·472	0·350	0·000	0·122	0·00	25·9	25·9	NNBY
	Asterias glacialis	Tube feet	0·081	0·022	0·000	0·059	0·00	73·0	73·0	NNBY
	Strongylocentrotus lividus	Jaw muscles	0·374	0·089	0·106	0·179	28·40	48·0	76·4	NNBY
Arthropoda	"Lobster"	Tail muscles	—	0·740	0·000	—	0·00	—	—	EE
	"Crab"	Claw muscles	—	0·480	0·000	—	0·00	—	—	EE
	Astacus fluviatilis	"	0·500	0·220	0·000	0·280	0·00	56·0	56·0	NNBY
Protochordata	*Balanoglossus salmoneus*	Proboscis and collar	0·280	0·160	0·060	0·060	21·4	21·4	42·8	NNBY
	Ascidia mentula	Muscle of atrial wall	0·007	0·005	0·00	0·002	0·0	22·5	22·5	NNBY
	Amphioxus lanceolatus	Whole body	—	0·570	0·33	—	37·0	—	—	EE
Vertebrata	"Dogfish"	Coracomandibular muscle	0·550	0·360	0·19	0·000	35·0	0·0	35·0	M
	"Dogfish"	Electrical organ	—	0·510	0·18	—	26·0	—	—	EE
	Torpedo marmorata	Dorsal muscle	—	0·251	0·374	—	60·0	—	—	B
	"Plaice"	Coracomandibular muscle	—	0·910	0·37	—	29·0	—	—	EE
	Cottus (sp.?)	"	—	1·300	0·13	—	9·0	—	—	EE
	"Frog"	Coracomandibular muscle	0·220	0·300	0·50	—	63·0	—	—	EE
	"Frog"	Sciatic nerve	—	0·100	0·07	—	32·0	—	—	EE
	"Snake"	Dorsal muscle	—	0·650	0·40	—	38·0	—	—	EE
	"Tortoise"	Hind-limb muscle	—	0·640	0·15	—	19·0	—	—	EE
	"Guinea-pig"	Gastrocnemius	—	0·580	0·22	—	27·0	—	—	EE
	"Rabbit"	"	—	0·260	0·62	—	70·0	—	—	EE
	"Rabbit"	Sciatic nerve	0·280	0·090	0·06	—	21·0	—	—	GW

Key to references. EE Eggleton & Eggleton (1928), NNBY Needham, Needham, Baldwin & Yudkin (1932), M Meyerhof (1928).
ML Meyerhof & Lohmann (1928 b), GW Gerard & Wallen (1929). B Baldwin (1932).

brought about entirely by means of cilia. Nevertheless, it must be remembered that the muscles of adult animals are derived from embryonic *Anlagen*, and that the phosphagen of the embryos may be associated with the embryonic cells from which the adult muscles will arise (see p. 81). Yet it should be mentioned that while the muscles of the adult sea-urchin contain arginine *and* creatine phosphagens, only the former is found in the plutei and gastrulae. Furthermore, with reference to the case of *Pleurobrachia*, it is only fair to point out that the evidence for the occurrence of arginine phosphagen here is that of only one experiment.

The idea that both ciliary and muscular activity may perhaps derive their energy from processes involving the Meyerhof cycle has crystallised out from the results of modern work on ciliary motion. Thus Gray (1928) has listed a number of variables in relation to which it is possible to demonstrate physiological parallels between ciliary and automatic muscular activities. Glaser (1925, 1926), making *Paramecium* swim along glass tubes, measured the temperature characteristic of the time taken to travel a measured distance, and from his results was led to believe that this ciliate makes use of the lactic acid cycle. Beutler has shown histochemically that the ciliated cells of *Actinia* contain a large amount of glycogen, while Boyland has made a direct attack upon the problem by estimating the glycogen of the ciliated gills of *Pecten*, but his results do not appear to have been fully published. The work on this subject has been fully discussed by Gray (1928).

This idea, that ciliary and muscular activity derive their energy from similar sources, is by no means altogether unjustifiable. But when it is realised that, as the work of Lundsgaard (1930 *a*, and later papers) has shown, a muscle is still capable of contraction after the lactic acid mechanism has been paralysed by means of iodo-acetic acid, and that according to the work of Warburg and his co-workers (1929) the lactic acid mechanism seems to be a property common to all animal cells, the observations just mentioned lose a very great deal of their weight. Needham, Robertson, Needham & Baldwin (1932) examined several species of flagellate and ciliate Protozoa, but failed to get evidence for the presence therein of either of the phosphagens at present known, or of any form of labile phosphate that might perform an analogous function.

Finally, it is interesting that the creatine of the electrical organs of *Torpedo* is present in the form of phosphagen (Kisch, 1930; Baldwin, 1932). It would be instructive to compare with these the corresponding organs of *Malapterurus* and certain other electrical fishes which are believed to be derived from glandular rather than from muscular sources. Table I, from Needham & Needham (1932), summarises the results of a number of workers on the distribution of the phosphagens.

(b) CREATINE AND PHOSPHAGEN IN ONTOGENY.

The ontogenesis of creatine itself has recently been discussed by Needham (1931). The presence of creatine in the unincubated avian egg has been a matter of debate, but there has never been much doubt that creatine is to be found in the organised tissues at a very early date. Tiegs (1924) was able to show that it is present

in the heart of the embryo chick on the fourth day, and in general tissues on the fifth. According to the results of Needham and Baldwin (1932) it seems that even earlier than this some creatine is already present in the form of phosphagen. At 70 hours, phosphagen was already present, and accounted for 20–30 per cent. of the sum of the free and labile phosphorus fractions in the whole embryo. The percentage P present as phosphagen rose to attain its maximum at about 90 hours, at which time, according to the recent work of Kuo (1932), active muscular movements, apart from the heart beat, are mainly being established. The absolute amount of phosphagen was rising rapidly at 70 hours and continued to rise *pari passu* with the weight up to the time of hatching. It is probable that the amount of phosphagen present at any given time is an index to the amount of muscle tissue present at that time. Mellanby (1908) and others have shown that the muscle creatine of kittens and other young animals continues to rise after birth, and does so until an adult level is reached, and it is probable that the same is true of the phosphagen content of the muscles of the newly hatched chick.

Similar considerations appear to hold in the case of the developing eggs of *Sepia officinalis*, a study of which was made by Needham, Needham, Yudkin & Baldwin (1932). In a curve showing the variation of the amount of arginine phosphate with the age of the embryo, a peak was observed on the 86th day, corresponding to the time at which the main muscle masses are being laid down. Arginine phosphate was also found in the plutei and gastrulae of *Strongylocentrotus lividus*, but not in the unfertilised eggs, though in *Sepia* phosphagen was found even in the infertile eggs. Creatine, as well as arginine, phosphate was found in the jaw muscles of the adult sea-urchin, but no signs of its presence in the plutei or the gastrulae were observed.

Practically nothing is known about the ontogenesis of phosphagen in mammals, but the following considerations of the maternal organism are of interest.

Dulière (1931) finds that the uterine muscle of white mice contains no phosphagen except during pregnancy. As pregnancy advances, the amount of phosphorus present increases by about 50 per cent., the extra phosphorus appearing as phosphagen. Shortly after delivery phosphagen is no longer to be found, but its components appear in the free state, and then diminish in amount as the uterus returns to its resting state. The ratio of the number of free creatine to the number of free phosphate molecules remains at unity throughout these processes. Dulière's results suggest the possibility of a periodic production and disappearance of phosphagen during the menstrual cycle in the human species, for uterine contractions are known to occur during the menstrual period.

How far these observations can help to explain the creatinuria of women, whether pregnant or non-pregnant, can be seen from the following summary of the work on the subject. Neither the creatine nor the creatinine contents of the blood are altered in menstruation, nor, except in primiparae sometimes, in pregnancy, parturition, nor the puerperium. It has been suggested that the creatinuria of women may possibly be related to the menstrual cycle, but on the whole the evidence seems against rather than in favour of this hypothesis. Creatine is regularly to be

found in the urine during the last month of pregnancy, and the excretion increases in amount till delivery. After parturition the rate of excretion rises still further, and more creatine is excreted than can be accounted for by the amount present in the gravid uterus, in spite of the fact that this organ contains more creatine, both relatively and absolutely, in this condition than in any other (see Beker, 1913). It has been suggested that the creatinuria which follows delivery may be due to the involution of the uterus, but creatinuria still occurs after hysterectomy. Hypertrophy of muscles other than the uterus during pregnancy, followed by a retrogression after parturition, might account for this creatinuria, or, on the other hand, it may be that creatine is produced at an unusually great rate at these times, or that there is a mobilisation of creatine from other parts of the body. But the reason for any of these changes is anything but clear. (A full presentation of the evidence bearing on the creatine metabolism of women is to be found in Hunter's monograph.)

III. RELATION OF PHOSPHAGEN TO MUSCULAR ACTIVITY[1].

In their first paper on the subject of phosphagen, Eggleton & Eggleton (1927 *a*) reported that the resting inorganic phosphate content of frog gastrocnemius is much lower than was previously believed, and of the order of some 20–30 mg. per cent. Determinations by older methods gave values of 90–100 mg. per cent., and the Eggletons showed that the difference between these values was due to the breakdown, during the earlier processes of estimation, of the very labile compound which they termed phosphagen. They showed that in rapidly induced fatigue the inorganic P is doubled, but that rather more phosphagen disappears than is necessary to account for the amount of free phosphate appearing. The phosphagen disappearing in activity was found to be resynthesised on recovery. When a muscle goes into rigor, the phosphagen disappears altogether, the inorganic phosphate rises to about four times its resting value, and the increase is more than can be accounted for by the phosphagen broken down. These early observations were considerably amplified and extended in a later paper on the physiological significance of phosphagen (1927 *c*), and at this time the authors were inclined to think that phosphagen was an ester of some carbohydrate, similar to, or perhaps the same as, the lactacidogen of Embden. The Eggletons suggested that this compound might enter into the carbohydrate cycle thus:

(*a*) Phosphagen → lactic acid + inorganic phosphate.
(*b*) Inorganic phosphate + glycogen → *X*.
(*c*) *X* → phosphagen.

They found that when a muscle was tetanised and most of its phosphagen was thereby removed, about two-thirds reappeared as inorganic phosphate, while rather more than one molecule of lactic acid appeared for every molecule of phos-

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[1] For a very complete survey of the subject of muscle chemistry the reader is referred to the monograph of D. M. Needham (1932) and to the more recent review by P. Eggleton in the present number of this *Journal*.

phagen broken down. No anaerobic resynthesis of phosphagen was observed, but when oxygen was admitted to the muscle, the phosphagen rapidly reappeared, while an exactly equivalent amount of inorganic phosphate was lost. The phosphagen was completely resynthesised in a short time, during which only a small amount of the lactic acid was oxidised away. Phosphagen was also broken down during anaerobic rest (Eggleton & Eggleton, 1928).

The influence of the fluoride ion was also studied. If a minced muscle were suspended in a bicarbonate buffer, all the phosphagen disappeared, giving place to free phosphate, and in equivalent amount. But in the presence of fluoride, although the phosphagen still broke down, there was no liberation of free phosphate. In both cases an equivalent amount of free creatine (estimated by the Walpole method) appeared. Now Embden & Lehnartz had already shown (1924) that in the presence of fluoride such a suspension showed a large degree of synthetic action, phosphate being apparently esterified, while Deuticke (1925) had found that this synthesis did not take place in extracts of rigorised muscles. These workers believed that free inorganic phosphate was involved in this synthesis, since they were unaware of the existence of phosphagen, but it seems likely, especially in view of Deuticke's result, that it is phosphagen itself and not free phosphate that is concerned. One might notice in passing that involuntary muscle is said (Eggleton & Eggleton, 1928) not to show this behaviour, probably because the amounts of phosphagen present in it are so very small. The Eggletons, finding that the ester formed in the synthesis to which we have referred was, like Embden's lactacidogen, hydrolysed by incubation in the bicarbonate buffer, and in the same way as was lactacidogen, concluded that the two substances were probably identical. This could be explained in the scheme of reactions already given, by the supposition that reaction (c) was inhibited by fluoride. We have already mentioned that the amount of phosphate corresponding to the phosphagen broken down in normal activity does not all appear in the free state, and the amount disappearing was traced to the formation of acid-soluble esters in the muscle extracts, while the apparent excess of phosphate appearing in rigor was later found to be due to the breakdown of other phosphorus compounds, notably pyrophosphate (Lohmann, 1928 a).

At this stage, however, Eggleton & Eggleton began a careful purification of their phosphagen and it soon became doubtful whether it was a carbohydrate ester after all (1927 b). Hearing of the work of Fiske & Subbarow (1927 a, b) they tested their product and found that, like that of Fiske & Subbarow, it contained creatine, and, as purification proceeded, that the creatine and phosphate were present in equimolar amounts. Fiske & Subbarow also found that the compound which they showed to be creatine-phosphoric acid was broken down in contraction and resynthesised in recovery, disappeared in rigor, and so on, in general agreement with the Eggletons' observations.

We must here diverge for a moment to discuss the part played by phosphagen in the thermal phenomena accompanying contraction. It had been shown by the work of Hill, Meyerhof, and their co-workers, that the heat set free in a contraction is liberated in two phases, one part during the actual contraction, while lactic acid is

being produced from glycogen, and the second during oxidative recovery, part of the lactic acid being burnt off and the rest being reconstituted into glycogen. Considering the heat of contraction, it was found by Meyerhof and others that 390 cal. were produced for each gram of lactic acid produced from glycogen in the muscle. The difference in the heats of combustion of these two compounds was 185 cal. (the glycogen being in the solid state and the lactic acid in solution). A further heat production arises from the neutralisation of the acid by deionisation of proteins and also to some extent by phosphate buffering. This process accounted for a further 80 cal. The remaining $390 - (185 + 80) = 125$ cal. remained unaccounted for. Following the isolation and identification of phosphagen, Meyerhof & Lohmann (1927) determined its heat of hydrolysis, and the ratio between the amounts of phosphoric acid and lactic acid produced, in unfatigued muscles, and found that for every gram of lactic acid formed, from 120 to 130 cal. were produced by the concomitant hydrolysis of phosphagen, thus accounting for the missing quota.

Meyerhof (1928) has shown that the arginine phosphagen of the invertebrates behaves in a similar manner. In *Pecten*, *Holothuria* and in the podaxonian worm *Sipunculus*, he showed that the phosphagen content was diminished by stimulation and reduced to zero by rigor. The free arginine was estimated in some cases, and was found equivalent to the phosphagen disappearing. The phosphagen content was also found to be below normal in animals in poor physiological condition. In the mantle muscle of *Sepia*, to take an interesting case, Meyerhof found that no phosphagen was present. Later, however, Needham, Needham, Baldwin & Yudkin (1932) investigated the case, and found abundant phosphagen in the fin, mantle, funnel and tentacle of *Sepia officinalis*, and so also in *Octopus vulgaris* (but see p. 99). But in one specimen of *Sepia*, which was floating on the surface of its tank, and which offered very little resistance to capture, only a small fraction of the usual amount of phosphagen could be found. It is therefore quite likely that Meyerhof's specimen was in a similarly advanced state of physiological decrepitude, and that his failure to find phosphagen here was due to this condition. In the same paper Needham, Needham, Baldwin & Yudkin describe the effect of electrical stimulation on *Nereis diversicolor* in an atmosphere of nitrogen; it was found that the major part of the phosphagen was thereby split up. In the jaw muscles of *Strongylocentrotus lividus* almost all the phosphagen (of both types) was found to be decomposed in heat rigor, while a specimen of *Balanoglossus* showed a similar behaviour.

Little or no work from this point of view seems to have been done on the influence of activity, fatigue, etc., on the smooth muscles of the gut and uterus, partly, no doubt, on account of the fact that they cannot be tetanised. Clark, Eggleton & Eggleton (1931) have investigated the case of the frog's heart, and found that "frog ventricles subjected to lack of oxygen showed a rapid loss of phosphagen, with a compensating increase in orthophosphate content. Both changes were quickly reversed on readmission of oxygen."

Finally, it should be noticed that the phosphagen of the electrical organ of *Torpedo* breaks down in activity (Kisch, 1930) and in "rigor" (Baldwin, 1932),

while the amount present is also considerably diminished when the animal is exposed to unfavourable conditions (Baldwin, 1932).

Thus there can be no doubt that the part played by phosphagen is no small or unimportant one, and the matter will be further discussed in the section on contraction without lactic acid production.

IV. CREATINE AND PHOSPHAGEN *IN VIVO*.

The removal of a muscle from the body necessarily involves a certain amount of stimulation, and a consequent breakdown of phosphagen. Eggleton & Eggleton (1929), and Dulière & Horton (1929) have found that, if freshly excised muscles are allowed to rest in water-saturated oxygen for some time before analysis, the concentration of phosphagen increases considerably. After a few hours of rest under these conditions, the muscles pass into a state of reversible inexcitability. In a case mentioned by Dulière & Horton, one such muscle was given 300 one-second tetanic stimuli at one-second intervals without producing the slightest effect upon the phosphagen content. In spite of this, however, the muscles could readily be revived by a short immersion in Ringer's solution, and after this treatment behaved in a perfectly normal manner. In view of the inexcitable nature of these muscles, it is unlikely that they would be appreciably stimulated by being ground up with trichloracetic acid, and it is therefore probable that the figures for their phosphagen contents would be nearer to the real resting value for the muscle *in situ*, though even in this case a certain amount of hydrolysis would be unavoidable in the extraction process. Estimations performed upon ordinarily treated muscles give values of the order of 30 mg. per cent. of free phosphate phosphorus, while Sacks & Davenport (1928), taking rather more than the usual care in the treatment of their muscles, which were gradually frozen *in situ* under amytal anaesthesia, obtained values of 20–30 mg. per cent., often as low as 21 or 22 mg. per cent., and regarded these as establishing "the normal range of inorganic phosphate" in the muscle *in situ*. Fiske & Subbarow (1929) have also obtained values of this order, but they point out that some loss of phosphagen is inevitable in the extraction. But the inexcitable muscles above mentioned give values as low as 10–20 mg. per cent. of phosphagen P. One might expect the muscle *in situ* to be in phosphate equilibrium with the blood. It has been known for some time that a muscle placed in Ringer's solution loses both phosphate (Embden & Adler, 1922) and creatine (Tiegs, 1925) faster in a fatigued than in a resting state, and an increase in the blood phosphate of athletes after a short spell of violent exertion has been recorded by Harvard & Reay (1926), while Embden & Grafe (1921) report that there is an increased excretion of phosphate by the kidney during the performance. All the evidence seems to point to a free diffusibility of phosphate through the cell wall. Stella (1928) has shown that a resting muscle immersed in well-oxygenated Ringer's solution is in osmotic equilibrium with about 8 mg. per cent. of phosphate, while the blood contains only a little less than this, viz. some 6 mg. per cent. It does therefore seem highly probable that the muscle is normally in phosphate equilibrium with the blood.

It has usually been held that there is normally a slight excess of creatine over free phosphate in the muscle. But it is well known that when phosphagen breaks down to give free creatine and phosphoric acid, a certain amount of the latter becomes esterified, and can no longer be regarded as "free." This process quite probably takes place in the muscle *in situ*, but that it takes place in the excised muscle is certain. If the process were not completely reversible in the excised muscle, it is evident that there would always appear to be some excess of free creatine, even if the two components of phosphagen were initially present in equivalent amounts. At any rate, the free creatine can be converted to phosphagen by placing the muscle in phosphate solution. Thus Nachmansohn (1929) finds that if a muscle be allowed to rest in $N/50$ phosphate solution at a pH of 7·2, the phosphagen content increases by 25 per cent., all the free creatine being converted.

It was for a long time believed that there is an excess of free creatine over free phosphate of the order of 30 mg. per cent. The experiments upon which this opinion was based were made using the method of Folin for the estimation of creatine. Involving as it does the unspecific reaction of Jaffé for creatinine, it was possible that some, at least, of the chromogenic material was not derived from creatine at all. In fact, it was shown by Baumann & Ingvaldsen (1916) that if the estimation of creatine by the Folin method were preceded by its isolation as creatinine (in the form of the potassium salt of the picrate) the values then obtained were always some 30 mg. per cent. lower. So it would appear not unlikely that the amounts of free creatine and of free phosphate ordinarily present are approximately equivalent, indicating the possibility that in the living, resting muscle *in situ*, all the creatine and practically all the phosphate may well be present in the form of phosphagen.

In this connection the work of Dulière (1929) must be mentioned. Working with muscles that had been allowed to rest in oxygen for some time before analysis, Dulière estimated the free and the combined creatine by a modification of Walpole's method, and also, and with confirmatory results, by the Folin method, after a good deal of the impurities had been removed. His method is a great deal more satisfying than those of earlier workers, and he found that when the muscles had been allowed to rest for a long time, the ratio of free phosphate to free creatine was, in one set of experiments 0·975, and in a second 0·94. When the muscles were in a less satisfactory state of rest, this ratio fell to 0·5–0·6, and, as we have seen, this decrease is to be expected on account of the esterification of free phosphate. The ratio of the combined, *i.e.* labile, phosphate to the combined creatine was 0·97, and was almost unaffected by the condition of the muscles. In resting, well-oxygenated muscles, the amount of free creatine was found to be as little as 50 mg. per cent. (equivalent to 12 mg. per cent. of P), a value confirmed by Eggleton's work on the subject. He found (1930) that a resting muscle is in osmotic equilibrium with a solution containing 80 mg. per cent. of free creatine, which corresponds to a value of 65 mg. per cent. (equivalent to 15·5 mg. per cent. P) in terms of the weight of the fresh muscle. In contrast to this value, Eggleton found that a fatigued muscle equilibrates with some 200–300 mg. per cent. of creatine in the external solution. Thus it would

seem unlikely that there is any marked excess of free creatine over free phosphate in the muscle *in situ*, for if this were the case, one would expect to find a marked loss of creatine by simple diffusion, for there is no evidence that the cell is impermeable to creatine.

But it is otherwise with phosphagen. This does not appear to be diffusible. Horton (1929) has found that the creatine-phosphoric acid isolated by Eggleton & Eggleton and others will pass freely through collodion membranes, although it never passes out from the muscle cell. On these grounds, the Eggletons (1929) have suggested that in the living cell the creatine-phosphoric acid must be linked up in some way with a colloid material, and so become non-diffusible. This indeed must be the case, for otherwise it is impossible to account for the observations of Folin & Denis (1912, 1914), Myers & Fine (1913) and others, that after the oral or intravenous administration of creatine there may be very considerable increases in the creatine content of the muscles. Folin & Denis themselves attributed their findings to the presence of muscle creatine as a complex, rather than in the free state. A number of workers have attempted to get the same results, and among these mention might be made of Fiske & Subbarow (1929), who tried injections of creatine, and of creatine mixed with potassium phosphate, without success. More recently the matter has been reinvestigated by Brown & Imrie (1931). In their experiments, creatine was introduced into the duodenum, the renal arteries being ligated to prevent excretion by the kidney. The creatine content of the muscle was regularly found to be increased, and with it the total acid-soluble phosphate. A fall in the excretion of phosphate by the kidney was also observed when this organ was allowed to operate, the effect being more marked when bigger doses of creatine were given. But their results lead them to remark that a certain amount of the creatine of the muscles seems to be present " not as phosphagen," but, as they unfortunately used the Folin method for their analyses, this statement must be taken with due consideration of the unspecific nature, already so frequently remarked upon, of the Jaffé reaction.

The general situation, then, is that the introduction of free creatine into the circulation may, under suitable conditions, be followed by an increase in the phosphagen content of the muscles, the requisite phosphates being removed from the blood, and non-diffusible phosphagen being formed in the muscles, while the removal of phosphate from the blood is compensated for by a diminished output by the kidney. The free creatine and the free phosphate of the muscle are probably small in amount and in osmotic equilibrium with those in the blood, while by far the greater part of both is present in combination as creatine-phosphoric acid, linked up to some colloidal moiety which renders it non-diffusible.

V. CONTRACTION WITHOUT GLYCOLYSIS.

A brief reference to the work of Lipmann & Meyerhof (1930) is made elsewhere in this discussion, but a closer examination is here desirable. By suspending a thin sartorius muscle in a bicarbonate solution, above which is an atmosphere containing CO_2, it is possible to obtain equilibrium between the CO_2 of the atmosphere and

that of the muscle itself. The equilibrium tension of CO_2 will then be related to the pH of the muscle by the Hasselbalch-Henderson equation:

$$pH = pK + \log_{10}\frac{[HCO_3']}{[CO_2]}.$$

A thin muscle such as the sartorius is preferred, since the equilibration of the muscle with the solution otherwise takes a very long time. Lipmann & Meyerhof used a modification of Warburg's manometric technique (1925) for the measurement of the CO_2 tensions, and examined the pH changes taking place in the muscle under a variety of conditions of stimulation. For a resting muscle there was very little change of pH in the course of time, but on stimulation there was a marked shift towards the alkaline side. For example, when the initial pH was about neutrality, the first tetanus led to an alkaline shift, and the second had the same effect. But the third resulted in a shift to the acid side. If the muscle was acid in the first place, the alkaline shift outlasted a greater number of stimuli, while, if it were alkaline at the beginning, the acid shift began at once with the first stimulation.

Some experiments were performed in which the muscle was taken at the moment when the first alkaline shift had just been compensated by the subsequent acid shift, *i.e.* when the pH had just returned to its initial value. On analysis, it was found that the following amounts of acid and base had been liberated:

$0{\cdot}59 \times 10^{-5}$ equivalents of acid from glycolysis,
$0{\cdot}50 \times 10^{-5}$ equivalents of base from phosphagen breakdown, and
$0{\cdot}06 \times 10^{-5}$ equivalents of base from ammonia formation.

The observed changes of pH could always be accounted for by the chemical changes known to take place in the muscle. During the early stages, *i.e.* the first two tetani, the phosphagen effect predominates, and afterwards the lactic acid effect is in predominance. It appears that the formation of lactic acid would be preceded by a breakdown of phosphagen in the normal muscle, since the pH of the latter is such that an alkaline shift would take place before the acid shift due to the formation of lactic acid became apparent. It seems then that the muscle has to reach a certain degree of alkalinity before any lactic acid is formed. So that in the early stages of activity, *i.e.* before the muscle has broken down enough phosphagen to reach the pH at which lactic acid formation commences, the energy must come from some process other than glycolysis, since during that time there is no glycolysis from which the energy could be provided.

Further evidence for the importance of phosphagen breakdown is to be found in the work of Nachmansohn (1928), who showed that in a non-fatigued muscle there is a very rapid anaerobic resynthesis of a part of the phosphagen broken down during a contraction. After two 5-sec. tetani, about 25 per cent. of the phosphagen broken down in the contractions was resynthesised in the 30 sec. immediately following the contraction. As fatigue advanced the amount of anaerobic resynthesis fell off, and by the time the phosphagen was almost exhausted the muscle would no longer contract in a normal manner, but showed a marked degree of contracture. These results

suggest that phosphagen breakdown is essential for normal contraction, and also that anaerobic resynthesis allows of a very much larger phosphagen breakdown, and consequent performance of work, than could otherwise be the case under anaerobic conditions.

In 1929 Lundsgaard (1930 a), while working on the effect of monoiodoacetic acid on animals, was struck by the fact that when the muscles went into rigor, as they do under the influence of this drug, they showed no sign of the acidity usually to be found in rigorised muscles. An examination of the muscles soon showed that no lactic acid had been formed in them. They were, in fact, alkaline, rather than acid. The gastrocnemius of a frog poisoned with iodoacetic acid was stimulated until no further response was obtainable, and in this way about 100–150 twitches were obtained. By taking a normal muscle and treating it in the same manner, a control was available, and on working up the two muscles for lactic acid and for phosphagen the following results were obtained:

Table II.

		Lactic acid	ortho-phosphate	Phosphagen
NORMAL:	Resting	25	21	61
	Working	84	29	46
POISONED:	Resting	16	29	57
	Working	15	28	0

The only possible conclusion is that of Lundsgaard himself, viz. that the energy for the contraction of the poisoned muscle must have been provided by the hydrolysis of the phosphagen which has disappeared. The amount of heat to be expected from the amount of lactic acid appearing in the normal muscle was calculated as 22·2 cal. per gram of tissue, while that for the phosphagen breakdown in the poisoned muscle was 22·7 cal. per gram. Again, the normal muscle after doing its work contained some 40 mg. more phosphagen phosphorus than the poisoned muscle, *i.e.* 40 mg. must have been synthesised by the normal muscle, requiring an energy expenditure of 16·5 cal. But the poisoned muscle formed no lactic acid, whereas that formed by the normal muscle corresponds to an energy production of 16·8 cal. The agreement between the calculations and the observations is in both cases remarkable. Later, Lundsgaard (1931 a) was able to show that crab muscles poisoned with iodoacetic acid behave very much as do frog muscles similarly treated, and that here again there is no formation of lactic acid. Although in this case the numerical agreement was not so good as in the case of the frog muscle, the same conclusions apply, viz. that the phosphagen of the normal muscle is resynthesised at the expense of glycolysis.

Thus, then, arises the conception of phosphagen hydrolysis rather than glycolysis as the energy source of the contraction process. Even if it is not the immediate source, it is at any rate a stage nearer than is lactic acid production. If this is indeed true one would expect to find the ratio

$$\frac{\text{tension} \times \text{length of muscle} \times \text{duration of tetanus}}{\text{phosphagen broken down}}$$

remaining constant in the poisoned muscle, and this is actually the case. In a remarkably striking figure, Lundsgaard (1930 b) showed the close proportionality between the phosphagen broken down and the tension developed. The lactic acid mechanism is therefore to be regarded as a system used for the resynthesis of phosphagen, rather than as the energy source itself. Whether the hydrolysis of phosphagen can be regarded as the immediate source of the contraction energy is still uncertain. Perhaps the energy from the hydrolysis is communicated directly to the mechanical systems of the cell, but on the other hand it is not impossible that there may be intermediate systems of a sort at present unknown.

In the poisoned muscles it was found that there was no longer any anaerobic resynthesis of phosphagen. Nevertheless, if the poisoned muscles were kept in oxygen, a much greater performance of work was possible than under anaerobic conditions. Since glycolysis appears to be completely inhibited by iodoacetic acid, it is evident that the energy for the phosphagen resynthesis which must take place here, must arise from a source other than glycolysis. It has been shown that there is a considerable amount of resynthesis of phosphagen in enzyme-containing muscle extracts, kept at a slightly alkaline pH, provided that these extracts also contain adenyl pyrophosphate (Meyerhof & Lohmann, 1931 a). The breakdown of this compound, giving rise to o-phosphate, ammonia, and inosinic acid, is associated with a heat-production of 170 cal. per gram of H_3PO_4 and could therefore furnish considerable amounts of energy for the resynthesis. If glycogen be added, the amount of synthesis is increased, presumably because a further store of energy becomes available. But glycogen alone produces no synthesis in the absence of adenyl pyrophosphate. Thus it seems not impossible that in the intact muscle the energy source for the anaerobic resynthesis of phosphagen may lie in the breakdown of adenyl pyrophosphate, this being in its turn reconstituted at the expense of glycolysis. Meyerhof & Lohmann (1931 a, b) have suggested that adenyl pyrophosphate is alternately broken down and resynthesised during the activity of normal muscle.

The lactic acid mechanism is not only slower in operation than the phosphagen hydrolysis, but is also less efficient. For, in a muscle which is made to perform a series of twitches, the amount of phosphagen present falls off, and at the same time the amount anaerobically resynthesised falls off also. The lactic acid part of the machine cannot, as it were, supply energy at so great a rate as it is set free by the phosphagen part. Thus eventually the amount of phosphagen present falls to zero, and there is no longer any resynthesis. By this time the muscle has reached an advanced stage of fatigue and can no longer perform normal contractions but always shows a marked degree of contracture.

It should be mentioned that the heat-tension ratio is, according to Meyerhof, Lundsgaard & Blaschko (1930, 1931), the same in iodoacetic acid muscles as in normal muscles, except in advanced fatigue, when it rises on account of the break-down of adenyl pyrophosphate and other compounds.

It might be remarked at this point that iodoacetic acid is not the only drug by means of which it is possible to demonstrate contraction without lactic acid formation. The fluoride ion has for some time been known to be capable of inhibiting

glycolysis, and Lipmann (1930) finds that it does so in the muscle cell, and that in muscles treated with fluoride there is no glycolysis during activity, and that the breakdown of phosphagen is very great compared with that of normal muscle: the ratio $\dfrac{\text{tension} \times \text{length} \times \text{duration of tetanus}}{\text{phosphagen broken down}}$ is the same as in the iodoacetic muscles. Ochoa (1930) has obtained muscles practically free from carbohydrates by means of insulin, and on activity these showed a much greater phosphagen break-down than normal muscles. Unfortunately, it is not possible to get the muscles entirely free from carbohydrates by this method, and the results are accordingly less clear cut than in the other cases. It is also interesting in this connection that, according to a private communication of Clark, Eggleton & Eggleton, the heart muscle of the frog also shows an inhibition of glycolysis under the influence of iodoacetic acid.

VI. (a) THE NATURE, PURIFICATION AND PROPERTIES OF CREATINE PHOSPHAGEN.

The isolation of creatine phosphagen depends upon the fact that its calcium and barium salts are soluble. Muscle tissues contain an enzyme which hydrolyses phosphagen with great rapidity, and this substance is therefore best isolated from deproteinated extracts such as are prepared by extraction with trichloracetic acid, usually 5–10 per cent., and preferably at 0° C., in order to keep the loss by break-down (which also takes place in acid solution) as small as possible. The free inorganic phosphate and a fraction of the hexose esters with insoluble calcium and barium salts can be precipitated at a pH of about 9, and separated by filtration or centrifugation. The filtrate or centrifugate now contains the phosphagen and the remaining fraction of the hexose esters, and at this stage only about one-half of the phosphorus present corresponds to phosphagen. These compounds can be precipitated by the addition of an equal volume of 90 per cent. alcohol, and by subsequent fractionation the phosphagen itself can be obtained in a fair state of purity. The separation and purification are described in detail by Eggleton & Eggleton (1927 *b*), Lohmann (1928 *b*), and Fiske & Subbarow (1929).

The physico-chemical properties of creatine phosphoric acid were described by Meyerhof & Lohmann (1927) in a preliminary announcement, and by Lohmann (1928 *b*). They have also been investigated by Fiske & Subbarow (1929). In the work of Lohmann, phosphagen was isolated as the barium salt, which on analysis proved to have the composition and molecular weight corresponding to the formula $C_4H_8O_5N_3PBa.3H_2O$, indicating that it is the salt of a compound containing creatine and phosphoric acid in equivalent proportions. The following formula was given by Fiske & Subbarow (1927 *a*, 1929) who isolated the calcium salt:

$$
\begin{array}{c}
\text{NH--P} \overset{\diagup \text{OH}}{\underset{\diagdown \text{OH}}{=\!\!O}} \\
\text{HN} \!=\! C \\
\text{N.CH}_3 \\
| \\
\text{CH}_2 \\
| \\
\text{COOH}
\end{array}
$$
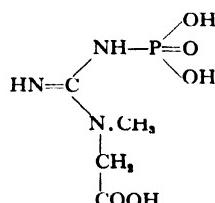
These authors remark that Michaelis and others (1896, 1903) have synthesised a number of compounds of the type $R.NH.POCl_2$, the acids corresponding to which were so unstable that they could not be isolated, with the exception of a few relatively insoluble aromatic derivatives. From this, and from the fact that they too showed it to contain equimolecular amounts of creatine and of phosphoric acid, Fiske &
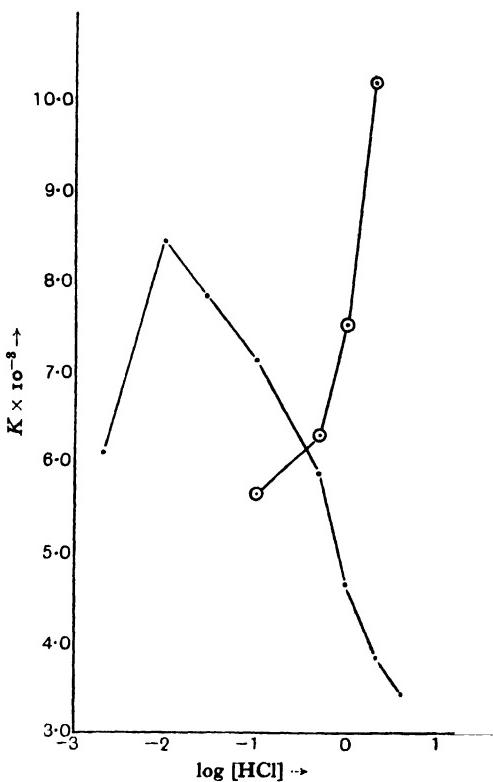

Fig. 1. Hydrolysis of phosphagen at 28° C. in HCl.

⊙—⊙ Creatine phosphoric acid [Lohmann, 1928 b].
•—• Argininine phosphoric acid [Meyerhof & Lohmann, 1928 b].

Subbarow give the above as the most probable formula, although some doubt still attaches as to its accuracy. Meyerhof & Lohmann (1927) are inclined to accept Fiske & Subbarow's suggestion.

The breakdown of phosphagen in acid solution was carefully investigated (Lohmann, 1928 b) and was found to be monomolecular in nature, and with a heat production of 150 cal. per gram according to Meyerhof & Suranyi (1927). Later work by Meyerhof & Lohmann (1928 c) has shown that this value is, however, too high and that 110–120 cal. is nearer the truth. The following data for the velocity constant of creatine phosphoric acid in acid solutions are taken from Lohmann

(1928 *b*), and were obtained on a preparation from rabbit muscle. For $M/80$ creatine phosphate in N HCl at 20° C., and after half an hour, $k = 5\cdot3 \times 10^{-3}$, while in the presence of $M/10$ molybdate, $k = 83\cdot2 \times 10^{-3}$, an increase of some 15 times. In crude preparations the addition of molybdate increases the velocity as much as 30 times. In an 80-hour experiment at 20° C. and in N HCl the mean k value found was $4\cdot84 \times 10^{-3}$. The effec tof the hydrogen ion on the hydrolysis was also investigated, and it was found that the velocity increases with the acidity. The following figures were obtained after 90 min. at 28° C. (see also Fig. 1):

<div align="center">Table III.</div>

[H˙]	0·2 N CCl₃COOH	0·1 N HCl	0·5 N HCl	1·0 N HCl	2·0 N HCl
$k \times 10^{-3}$	4·99	5·65	6·3	7·5	10·2

In order to show that the phosphagen extracted was the same as the substance present in the fresh extracts, the following solutions were prepared and gave the appended k values at 20° C.:

(*a*) A solution of the isolated product in 0·2 N trichloracetic acid,

$$k = 4\cdot14 \times 10^{-3} \quad \text{(7 experiments).}$$

(*b*) A fresh trichloracetic extract, adjusted to a normality of 0·2,

$$k = 3\cdot75 \times 10^{-3} \quad \text{(6 experiments).}$$

(*c*) An aqueous extract. This was left at pH = 8·8 in a carbonate buffer for 2 hours at 37° C., deporteinated with trichloracetic acid, and adjusted till the strength was 0·2 N,

$$k = 3\cdot87 \times 10^{-3} \quad \text{(7 experiments).}$$

It will be seen that the agreement between the three sets of experiments is very good.

It might be remarked that the k values were calculated from the equation for a monomolecular reaction, viz.

$$k = \frac{1}{t} \log_e \frac{a}{(a-x)}$$

(a = initial amount of phosphagen, x = amount broken down at time t, t being usually expressed in minutes). The logarithm is to the base e. The majority of Lohmann's figures are given in terms of common and not natural logarithms, but those quoted to show the effect of pH on the velocity constant appear to have been calculated in terms of the natural logarithms; the remainder are here recalculated in the same terms. This type of confusion is surely unnecessary, and is easily avoidable. Obviously some standard method of calculation must be employed, and it would seem rational to use the natural logarithms since it is in terms of these that the reaction equation is derived. Needham, Needham, Baldwin & Yudkin have recalculated (1932) a number of k values for unpurified extracts and expressed them in graphical form as a function of acidity.

Fiske & Subbarow (1929) have also worked on the acid hydrolysis of phosphagen obtained from cat muscle and, to add to the confusion already existing, expressed

their results in terms of the hour as time unit instead of the minute, and also used the common logarithms. Their values have been recalculated to compare with those of Lohmann, and are given below.

Table IV.

[H$^{\cdot}$]	0·5 N	0·1 N
$k \times 10^{-3}$	6·25	5·87

pH	3·0	3·4	3·8	4·2	4·6	5·0	5·4	5·8	6·2
$k \times 10^{-3}$	5·00	4·83	4·29	4·03	2·65	1·38	0·62	0·22	0·058

The hydrolysis of phosphagen by the muscle enzymes has been investigated by Meyerhof & Lohmann (1928 b), using phosphagen from frog muscle. The watery extracts prepared by the method of Meyerhof (1926) contain the lactic acid enzyme, and contain also an enzyme which rapidly hydrolyses phosphagen. Fifteen minutes after the preparation of such an extract, more than 60 per cent. of the phosphagen has been broken down, but the breakdown can be considerably retarded by the addition of glycogen or starch, especially in weakly alkaline solutions, acid-stable phosphate esters being simultaneously produced. That the breakdown is indeed enzymic is clear from the following facts:

(1) Boiled muscle extracts have no effect upon purified phosphagen, and the power even of unboiled extracts falls off in an hour or so at room temperature.

(2) The rate of hydrolysis of added phosphagen depends on the dilution of the enzyme-containing extract.

(3) Like the hydrolysis of the hexose phosphates, that of phosphagen by muscle extracts is inhibited by the addition of fluoride, a rapid esterification of phosphate taking place at the same time.

(4) The rate of splitting of phosphagen by muscle enzymes is maximal at pH 6·4–7; below pH 6·4 the rate falls off somewhat but the effect of alkalinity is much more striking. At pH 8·5 the rate of hydrolysis is already almost zero, and the inhibition is reversible over short periods of time. Beyond pH 8·5, if glycogen or starch be added, an actual synthesis of phosphagen takes place, though this effect is not very long lived. The amounts of phosphagen appearing, and of free creatine disappearing are equivalent, and the amount and rate of the synthesis can be increased by the addition of creatine. The synthesis is also favoured by an initially low concentration of phosphagen, such as can be induced by allowing the fresh extract to stand at room temperature for 30 min., while most of the phosphagen originally present decomposes. It might also be remarked at this point that the high pH necessary for the resynthesis can hardly be required when the intact muscle resynthesises phosphagen, for Nachmansohn has found that if an intact muscle be allowed to rest in $M/50$ phosphate solution at a pH of only 7·2 there is a synthesis of phosphagen (1929).

The breakdown of phosphagen and the production of lactic acid can be shown to be quite independent processes, although the fresh extracts contain both the enzymes. Carbohydrate-free muscle juice will split phosphagen readily and at the usual rate, while the purified lactic acid enzyme obtained by the method of Meyer (1928) produces lactic acid from glycogen in the complete absence of any phosphagen.

The heats of hydrolysis of both creatine and arginine phosphoric acids have been determined by Meyerhof & Lohmann (1928 *c*), and their data are given in Table V. We have already seen that the value for creatine phosphagen determined by Meyerhof & Suranyi was too high, and that the corrected value of 100–110 in neutral solution has been shown by Meyerhof & Lohmann (1927) to be sufficient to account for the apparent deficiency in the termal balance sheet for the contraction phase of muscular contraction.

We must now pause for a moment to consider the effect of the dissociation of phosphagen upon the pH of the contracting muscle. In this connection, Meyerhof & Lohmann (1928 *c*) have determined the electro-titration curves of both phosphagens, and compared with them the corresponding curves for their components in equimolar mixtures. They found that at the pH of normal muscle the mixture of creatine (or arginine) and phosphoric acid, which results from the dissociation of phosphagen, has a very marked buffering power. Phosphagen itself is quite unbuffered at this pH, and consequently the decomposition which takes place in contraction provides the muscle with an additional mechanism for the preservation of its pH. Fiske & Subbarow (1927 *a*, 1929) have also determined the electro-titration curves of creatine phosphoric acid and its components, and concluded that the dissociation must lead to the liberation of a large amount of free base, and that the muscle pH would therefore be shifted towards the alkaline side. They appear to have overlooked the buffering power of the creatine and phosphoric acid mixture. It was formerly believed that the liberation of lactic acid which takes place in the contraction process must lead to a shift of the pH of the muscle towards the acid side, but it is now clear from the work of Lipmann & Meyerhof (1930) that this is not the case. The ammonia liberation from adenylpyrophosphate undoubtedly plays a certain part in the neutralisation of the effect of lactic acid (see p. 88), but it seems probable that the main part is that played by the buffer mixture resulting from the phosphagen breakdown.

In conclusion, mention must be made of the "model" phosphagen prepared by Meyerhof & Lohmann (1928 *c*), viz. amino-phosphoric acid,

$$H_2N.P{=}O \begin{matrix} \nearrow OH \\ \searrow OH \end{matrix} \ .$$
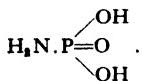
This substance was synthesised, and its behaviour studied. It was split by the enzymes which attack creatine and arginine phosphoric acids, and in general behaved like these compounds. Its heats of hydrolysis and its electro-titration curves were determined, and the heats of dissociation and pK values are given, together with those of the two phosphagens, in Table V.

Table V.

(Data from Meyerhof & Lohmann, 1928 c.)

Compound	Heat of hydrolysis, cal.		pK values		
	Acid	Neutral	Acidic		Basic
Creatine phosphoric acid	120–130	100–110	2·7	4·5	—
Arginine phosphoric acid	110–120	80–100	4·5	9·6	2·8
Amino-phosphoric acid	160	150	2·8	8·2	—

(b) THE NATURE, PURIFICATION AND PROPERTIES OF ARGININE PHOSPHAGEN.

The presence of a new amino-phosphoric acid in crustacean muscle was reported by Meyerhof & Lohmann (1928 a). The amounts of this substance to be found in crab muscle were of the same order as the quantities of creatine phosphagen to be found in vertebrate muscle. Like creatine phosphoric acid, it breaks down in contraction and is resynthesised on recovery. The compound in question proved to be arginine phosphoric acid. Kutscher (1914 and later papers) had already suggested that arginine plays in the invertebrates a part analogous to that of creatine in the vertebrates, and believed that in the latter creatine arises as a metabolic product of arginine.

In general it may be said that the properties of arginine phosphoric acid resemble those of the creatine compound. Both have soluble calcium and barium salts, and can be prepared from muscle extracts by similar methods. Meyerhof & Lohmann (1928 a, b) separated and purified the phosphagen of crab muscle. It was shown to contain arginine and phosphoric acid in equimolecular proportions and to contain a free (NH_2) group in the molecule (determined by the method of van Slyke), while the molecule was not attacked by arginase. These facts, and the molecular weight which was determined at the same time by these authors, agree with the formula

$$HN{=}C \begin{array}{c} \diagup NH{-}P{=}O \diagup ^{OH} _{\diagdown OH} \\ \diagdown NH \\ | \\ (CH_2)_3 \\ | \\ CH(NH_2) \\ | \\ COOH \end{array}$$
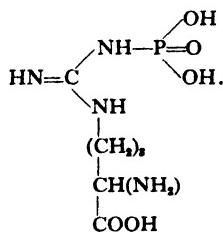
The conditions of acid hydrolysis were also investigated, and it was found that whereas creatine phosphagen breaks down more rapidly with increasing acidity, the velocity constant for arginine phosphagen has a maximal value at a hydrogen-ion concentration of about $N/100$. Also, while the breakdown of creatine phosphoric acid is accelerated by the presence of molybdate, that of the arginine compound is retarded some 30 times in purified preparations, or rather less in unpurified extracts.

This molybdate effect is probably responsible for the fact that this new phosphagen was not discovered by the Eggletons in their comparative studies (1928). In strongly acid, protein-free extracts, the arginine compound would break down rather more slowly than the creatine compound in any case, but in the presence of molybdate—and molybdate was present in the method employed by the Eggletons—not only would the breakdown of the creatine compound be accelerated some 30 times, but that of the arginine compound would at the same time be retarded another 30 times, thus making a relative difference of about 900 times. This effect of molybdate is obviously of great value in the separate estimation of the two phosphagens.

Meyerhof & Lohmann (1928 *c*) give a curve to show the variation of the *k* of arginine phosphoric acid with changing normality of HCl, and this curve has been plotted in a modified form in Fig. 1. The values of *k* have been recalculated in terms of natural logarithms, and together with them Lohmann's (1928 *b*) values for creatine phosphoric acid are also plotted for comparison. We have already referred (p. 96) to the heat of hydrolysis and to the electro-titration curve of arginine phosphoric acid.

Meyerhof & Lohmann (1928 *b*) have shown that arginine phosphagen plays in the claw muscles of *Astacus fluviatilis* a part similar to that of the creatine phosphagen of vertebrate muscles. The claws were chosen as having the advantage that symmetrical claws can be used for the initial and final estimations. The arginine phosphagen was estimated as phosphate after incubation of suitable extracts for 15 hours at 37° C. (without the addition of molybdate). This temperature was later abandoned in favour of 28° C., since Lohmann (1928 *a*) finds that a considerable amount of pyrophosphate may break down under the conditions first used.

Arginine phosphagen breaks down in activity and also, though more slowly, in anaerobic rest, and varies with the lactic acid production just as does creatine phosphagen in frog muscle. The phosphagen is also resynthesised during recovery. In enzyme-containing extracts, prepared according to the method of Meyerhof (1926) with 1 per cent. KCl, the phosphagen is broken down very rapidly, giving rise, as in the frog muscle extracts, to esters. In general, the breakdown and resynthesis of arginine phosphagen by the muscle enzymes goes on very much as does that of creatine phosphagen. In weakly acid solutions, the hydrolysis by the enzymes is rather slower than is that of creatine phosphagen, and the synthesis takes place more readily, beginning even at neutrality. The synthesis can be hastened and its extent increased by the addition of glycogen, starch, arginine or free phosphate, and is also favoured by a low initial phosphagen content in the solution. The change in the concentration of free arginine was followed by means of arginase, which splits off urea, this being decomposed by urease and the ammonia set free being estimated by Nesslerisation after being distilled off by the method of Parnas & Klisiecki (1926). Lastly, it must be mentioned that arginine phosphagen is split by the enzymes of frog muscles.

Meyerhof (1928) has studied the phosphagen of a number of invertebrates with a view to discovering whether one type of phosphagen is to be found in all invertebrates, since he had suggested that one might expect to find a series of different

phosphagens in the different groups of the animal kingdom. The data in Table VI are taken from his paper and show that the phosphagens of several different invertebrates show substantially the same behaviour under a variety of conditions. (The values for k are in terms of natural logarithms. All experiments at 28° C.)

Table VI.

(i) *Pecten jacobaeus* (90 min.).

HCl normality	0·002	0·01	0·1	0·4	2·5
$k \times 10^{-3}$	0·678	3·34	5·43	5·07	4·49

(ii) *Pecten jacobaeus* (120 min.).

HCl normality	0·002	0·01	0·1	1·0	0·1 + 0·3 % Mo.
$k \times 10^{-3}$	3·15	5·5	7·6	5·1	0·78

(iii) *Holothuria tubulosa* (120 min.).

HCl normality	0·0033	0·01	0·033	0·1	1·0	0·1 + 0·3 % Mo.
$k \times 10^{-3}$	2·79	5·51	6·06	7·73	4·97	0

(iv) *Sipunculus* (120 min.).

HCl normality	0·0033	0·01	0·1	1·0	0·1 + 0·3 % Mo.
$k \times 10^{-3}$	4·38	5·58	5·94	4·95	0·65

It will be seen that there is a considerable amount of variation in the k values at low acidities, and this Meyerhof attributes to different degrees of buffering in the different extracts. There is, however, a definite maximal value of k in $N/10$ solutions, while the purified product, and likewise fresh crab-muscle extracts, showed the maximum at $N/100$. Meyerhof attributes this difference to the presence of inhibitory materials in the extracts. Here, as in crab preparations, the addition of molybdate produces a very marked inhibitory effect, and the resemblance to the behaviour shown by preparations of crab muscle is close enough to demonstrate that the phosphagen here present is indeed the arginine compound. The probability is even greater in view of the fact that for every molecule of phosphagen disappearing a molecule of arginine was set free. This was found to be the case in extracts from *Pecten*, *Holothuria*, and from *Sipunculus*, the arginine being estimated by first splitting it by means of arginase prepared according to the directions of Edlbacher (1925), decomposing the liberated urea by means of urease, and estimating the ammonia set free by Folin's method (1926) by Nesslerisation.

Arginine has been isolated from a number of invertebrates in the form of its salts by Kutscher and his co-workers (1931), including Arthropoda, Mollusca,

Vermes, and Echinodermata, and it seems likely that in these animals it is present in the form of arginine phosphate. But it should be remembered that there is the possibility that other phosphagenic substances, derived from bases other than creatine or arginine, may well occur in the invertebrate phyla. In the work of Needham, Needham, Baldwin & Yudkin (1932), with the exception of a ctenophore, the coelenterates examined gave only very dubious results, and there is no doubt that they are worthy of closer attention. It is perhaps significant that the sponge, *Geodia gigas*, contains no arginine, its place being taken by guanidine and agmatine (Ackermann, Holtz & Reinwein, 1924; Holtz, 1924), while, according to Iseki (1931), *Octopus* muscle contains no arginine, but does contain a substance whose reactions and analysis indicate that it is a methyl agmatine. Corresponding to this, it has recently been shown by Baldwin (1932) that the phosphagen of *Eledone moschata* is, in all probability, not arginine phosphate. Its velocity constant for acid hydrolysis is minimal instead of maximal at pH 1, while molybdate produces an inhibition of only 3–4 times instead of 30. *Pecten*, however, contains the arginine compound, according to Meyerhof (1928), which is interesting in view of the generally accepted belief that the lamellibranchs left the main line of evolution at a date much earlier than that at which the Cephalopoda and Gastropoda branched off.

VII. THE ESTIMATION OF PHOSPHAGEN.

The acid-soluble phosphorus of the muscle can be resolved into the following fractions:

      (i) Initially present inorganic orthophosphate.
      (ii) Adenyl pyrophosphate.
      (iii) Phosphagen phosphate.
      (iv) Hexose phosphate.

At pH 9 the calcium and barium salts of the first two fractions are very insoluble, while those of the others are soluble. The phosphate of the adenyl pyrophosphate is estimable in two parts, firstly, the two atoms of pyrophosphate phosphorus which are relatively easily hydrolysed and, secondly, the o-phosphate atom of the adenylic acid which is only hydrolysed with difficulty. The most satisfactory methods of estimation as yet devised depend upon the relative solubilities of the calcium or barium salts of the different fractions.

The most suitable agent for the extraction of the acid-soluble phosphates is trichloracetic acid, by means of which a protein-free extract is readily obtainable. Since both the phosphagens are readily hydrolysed in acid solution, it becomes necessary to prepare the extracts at a low temperature, and to filter and neutralise the extracts as soon as possible. As a rule 5 or 10 per cent. trichloracetic acid is employed, and the extraction is carried out in cooled vessels, with cooled acid, by grinding up thoroughly with ice-cold quartz sand. The grinding must be very thorough, and according to Eggleton & Eggleton (1929) the extraction is then almost instantaneous. 10 c.c. of acid are usually suitable for the extraction of each gram of tissue, and as soon as the grinding has been carried out the extract must be

separated from the muscle debris by filtration or centrifugation. Eggleton &
Eggleton (1929) recommend the use of both these operations as being more rapid
than either alone, but the author prefers to filter the extract through asbestos under
pressure by means of a small cooled Gooch crucible, the extract being received in
a cooled tube. Before proceeding to discuss the separation methods to which we have
briefly referred, it is desirable to turn for a moment to a consideration of the
extrapolation method elaborated and used by Eggleton & Eggleton (1929).

In this method, as in the separation methods, the phosphagen is estimated in
terms of the o-phosphate liberated when the phosphagen itself decomposes. For
this purpose, Eggleton & Eggleton employ a modification of the Briggs method
(1922) for the estimation of phosphate. An acid solution of ammonium molybdate
is added, and the phosphate present combines with this to form a phosphomolybdic
acid, which, on reduction by a suitable reagent, gives a blue compound, by means of
which the colorimetric estimation of the phosphate is possible. Eggleton & Eggleton
use the original Briggs reducer for this purpose, namely o-quinone. According to
Fiske & Subbarow (1925) this reagent is liable to be influenced by a large variety of
substances which are of common occurrence in biological materials. The effect of
these substances is to cause a considerable retardation of the colour development, and
this, though of little importance in the separation methods, is, as will be seen, liable
to be a source of considerable error in the extrapolation method. In this method,
the rate of colour development of a suitable quantity of extract to which the colour
reagents have been added is compared with that of a similarly treated standard
solution made up at the same time. The standard is read against the unknown in the
colorimeter (the reverse of the usual procedure) and the readings are plotted against
the time. When this is done, the points lie on a straight line for the first 8 min., so
that, by extrapolation backwards to zero time, a value can be obtained for the
amount of inorganic phosphate present at the beginning. 50 per cent. of the
phosphagen breaks down under these conditions in the first 8–10 min., and the
breakdown is complete in about 1 hour. A fresh reading taken at this time gives the
final value for the phosphate, and from this is subtracted the initial value, giving the
amount which has been liberated by the phosphagen broken down. Eggleton &
Eggleton (1929) find that this method gives results in excellent agreement with those
obtained by their separation method, but although this is the case when frog
muscles are used it by no means follows that the method can be indiscriminately
applied to all muscle extracts unless they are shown to contain no substances capable
of hindering the rate of colour development. But for the estimation of phosphagen
in tissues known to contain no inhibitory materials, the method has the double
advantage of quickness and convenience. It cannot, however, be used for the
estimation of arginine phosphagen, since the breakdown of this compound is
hindered by molybdate, whereas that of the creatine compound is accelerated, the
resultant difference in rate being in some cases as great as 900 times. Fiske &
Subbarow (1925) suggest the use of 1-2-4-amino-naphthol sulphonic acid in place
of o-quinone as the reducing agent, since they believe that it is less liable to be
influenced by the presence of extraneous material.

In the separation methods the insoluble calcium and barium salts of the free phosphate and of the adenyl pyrophosphate are removed by precipitation with calcium or barium. Fiske & Subbarow (1925) neutralise their filtrates to pH 9 (faintly pink to phenolphthalein) by means of saturated caustic soda solution (which, like all the other reagents employed, must, of course, be free from phosphate), and then precipitate by the addition of a 10 per cent. solution of calcium chloride which has been saturated with calcium hydroxide. The precipitate is then spun down as rapidly as possible, and the supernatant liquid is poured off, the precipitate being washed with a suitable mixture of water and the calcium solution. The precipitate is again spun down and the washing added to the previous centrifugate. To the combined centrifugates, which contain the phosphagen, an acid molybdate solution is added, and the whole is left for 15–20 min., the reducing reagent added, and after another 15 min. to allow of full colour development a comparison is made with a standard phosphate solution. The precipitate is dissolved up in as little dilute sulphuric acid as possible, and to the solution the molybdate and sulphonic reagents are added, a comparison being made after 15 min. By a simple extension of the method the hexose phosphates of the centrifugate can also be estimated after a wet incineration (see Eggleton & Eggleton, 1929), while the pyrophosphate of the precipitate can be estimated after heating in normal acid for 7 min. in a boiling water bath, a process which hydrolyses the pyrophosphate without causing any appreciable hydrolysis of the other phosphorous compounds present. By wet incineration of the solution of the precipitate the total phosphate in it can also be determined, and corresponds to the sum of the inorganic phosphate initially present, the pyrophosphate from the adenyl pyrophosphate, and the o-phosphate of the adenylic acid itself.

These extensions of the separation method are described by Eggleton & Eggleton (1929) as extensions of their own separation method. In this they neutralise their extracts and also precipitate the insoluble phosphates by the addition of solid baryta. Apart from this and the fact that they still use o-quinone as reducing agent, their method is essentially the same as that of Fiske & Subbarow. Probably the use of 1-2-4-aminonaphthol sulphonic acid is to be preferred here as well as in the extrapolation method, since it saves a good deal of time in the colour development, a point of importance when a large number of estimations have to be carried out.

Finally, mention should be made of the method employed by Meyerhof (1928, 1930) and other German workers, in which free inorganic phosphate is precipitated by the addition of an ammoniacal solution of magnesium citrate.

Neither of the separation methods described comprises any method for the estimation of arginine phosphagen. This compound may be estimated by allowing a portion of the extract to stand overnight in $N/10$ acid at 28° C. The increase in phosphate content then corresponds to arginine phosphagen, since according to Lohmann (1928 a) there is no breakdown of pyrophosphate under these conditions. As we have already seen, arginine phosphagen and creatine phosphagen are seldom to be found together, but in the few cases where this is so both phosphagens will be accounted for in the extract which has been left overnight under these conditions,

the creatine phosphate can then be separately estimated by one of the methods previously described, and the amount of arginine phosphagen arrived at by subtraction.

The amounts of the phosphagens may also be estimated by determination of the amounts of creatine or arginine liberated when the phosphagens themselves decompose. By means of Walpole's method (1911), which involves the condensation of creatine with diacetyl, the free creatine of a solution or extract can be estimated, since Eggleton & Eggleton (1928) find that the reaction is not given by combined creatine in the form of phosphagen. The chemistry of the Walpole reaction has recently been worked upon by O'Meara (1931) and A. Roche (1932). The Walpole method is rather more difficult to handle than is Folin's (1926), but the latter, being based upon the Jaffé reaction, is liable to give rather high results on account of its lack of specificity. Sugars must be removed before estimation, and this may conveniently be done by the copper-calcium method given by Meyerhof (1930). The solution is then heated in the autoclave at 120° C. till the creatine is converted to creatinine which is then estimated by the Folin method. It is preferable to isolate the creatinine as its picrate before proceeding to the estimation, and this may be done by the method of Baumann & Ingvaldsen (1916). The chemistry of the Jaffé reaction has been worked upon by Greenwald & others (1924, 1925).

Arginine has been estimated by Meyerhof (1928) and Meyerhof & Lohmann (1928 a) and others by means of arginase. The enzyme is prepared from guinea-pig liver according to the directions of Edlbacher (1925), and is used to convert the arginine into urea and ornithine. The urea is then broken down by means of urease from the Jack or soya bean, and the ammonia so set free is distilled off, for which purpose the method of Parnas & Klisiecki (1926) is convenient; the ammonia is then estimated by Nesslerisation, according to the directions of Folin (1926). The method is a very useful one but the blanks on the reagents and enzyme preparations are usually so large in proportion to the amounts of arginine to be estimated that the errors are very considerable. Nevertheless, the method has the advantage of a high order of specificity.

Another method, of great sensitivity, is Weber's modification (1930) of the reaction of Sakaguchi (1924). The specificity here is not so great, since substances of the general type

$$HN{=}C\big\langle{}^{NH.X}_{NH_2}$$
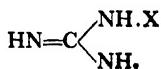
give the reaction, which is therefore also positive with glycocyamine, α-guanidine-butyric acid, etc.

These methods suffice to allow of accurate estimations of the two phosphagens at present known, and are capable of extensive application, and with their aid a wide field still remains to be explored.

VIII. SUMMARY.

1. The creatine of vertebrate muscle is mainly present in the form of a compound with phosphoric acid.

2. This labile compound is intimately associated with muscular contraction. It is broken down in activity and reconstituted during rest, and is a more immediate energy source than is glycolysis. It is also broken down under conditions which are unfavourable to the organism as a whole.

3. Similar considerations appear to hold in the case of the electrical organs of certain fishes, and may possibly hold for ciliary motion also.

4. The creatine of the vertebrates is replaced, in many if not in all invertebrates, by arginine, which also forms a labile compound with phosphoric acid, and this is of a physiological significance exactly parallel to that of creatine phosphoric acid.

5. The preparation, properties, and physiological behaviour of these compounds are described and discussed, while the methods in common use for their estimation are also described.

6. Guanidine derivatives other than creatine and arginine may also behave in the same way, giving phosphagenic compounds of similar functional importance. One such compound appears to be present in cephalopod muscle.

7. Creatine phosphate is practically confined to the vertebrates, whereas arginine phosphate is never found in members of that phylum. Both compounds have been found together in an echinoid and in an enteropneust. The Echinoderm-Enteropneust theory of vertebrate affinity, previously postulated on purely morphological grounds, seems thus to find new support on biochemical grounds.

8. The Cephalopoda appear to contain a phosphagen whose base is not arginine, whereas arginine phosphate appears to be present in the Lamellibranchiata. This is possibly of evolutionary significance, since the Lamellibranchiata are believed to have branched off from the main line of evolution at a date considerably earlier than that of the divergence of the other Mollusca.

9. Protozoa appear not to contain phosphagen, but with a possible exception in the case of the Coelenterata, the Metazoa all make use of some phosphagen compound.

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PALINGENESIS AND PALAEONTOLOGY

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(With Eight Text-figures.)

CONTENTS.

I. INTRODUCTION.

SINCE the days when Haeckel first propounded his famous Biogenetic Law, there has been very little agreement over its main tenet—that the individual animal, in its life history from zygote to adult, passes through a series of stages which, at least in some measure, mirror the various stages passed through in its phyletic evolution: that ontogeny, however distortedly, repeats phylogeny. While the majority of palaeontologists appear to have placidly accepted the principle with few qualms and without bringing to bear upon it any sort of critical analysis, using it indeed with facile ease to establish the most speculative lines of descent, the zoologists on the contrary have tended generally to repudiate its principal contentions and even to deny it altogether. Thus recently the subject has been revived in acute form by Garstang (1922, 1929) and de Beer (1930), who, from a study of embryonic and larval forms, and on other grounds, assert that, far from ontogeny being a condensed replica of phylogeny, the reverse is the case, and "Ontogeny does not recapitulate Phylogeny; it creates it" (Garstang, 1922, p. 82) (see also Shumway, 1932). Such a consideration, if true, would not merely violate the cherished faith of complacent palaeontologists: it would have a far more practical effect in shattering innumerable supposed phylogenies and lineages based on a Haeckelian foundation, and would entail the replacement of what is now considered to be in many instances (and

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ideally always) a genetic classification of fossils by a classification essentially based upon comparative morphology, with its attendant dangers.

It would seem, however, that to a considerable degree the confusion and disagreement are due to a lack of appreciation on both sides of the main grounds of contention. It is perhaps true that palaeontologists are inclined to forget that fossils are but the dry bones—often a very small and unimportant portion—of the animals which built them; they are never brought into contact with the more "vital" protoplasmic parts; they are therefore less disposed either to consider or to understand criticisms based on such foundations as the rates of hormone reaction or the hereditary transmission of internal factors. On the other hand, the zoologists do not appear to be aware of the weighty cogency attaching to the fossil evidence, which is a matter of course to the palaeontologist. In particular, the zoologists seem prone to live in the present, and to interpret phylogeny in terms of a morphologically graded series of more or less complicated contemporaneous types; whereas it need scarcely be emphasised that the ancestry of any particular animal is not to be found in stoppered bottles of formalin, but (such as it is) in the rocks.

Several palaeontologists have endeavoured to throw light on the subject by reference to the fossil evidence, but as their conclusions have been called in question, and are perhaps not entirely unequivocal, it may not be out of place to make a fresh attempt upon the problem by an examination of certain selected instances of seemingly indubitable animal evolution as displayed by palaeontology. Not the least merit of such a study lies in the relative simplicity of fossil structure, whereby emphasis is thrown upon, and confined to, the changes in only a few morphological skeletal characters, and the confusion of irrelevant phenomena, so liable with active living forms, is largely avoided (see, in this connection, Cumings, 1910). No endeavour to give a comprehensive review of the subject has been made; still less to answer all the arguments put forward in contradiction to Haeckel's doctrine. But it is suggested after a consideration of the evidence that at least in some cases there can be little doubt that ontogeny recapitulates phylogeny in the full sense of ancestral adult representation. The cause of this recapitulation is a question scarcely to be answered by excursions into the dead world of fossils; it is sufficient, so far as the palaeontologist is concerned, that the fact of recapitulation is established.

II. CRITIQUES OF THE PROBLEM.

During the early years of last century, in the days before the general acceptance of the evolutionary concept, the Aristotelian idea of a graduated Scale of Beings, from the lowliest "corals, coralloids, and lithophytes" to the highest, Man, seized the imagination of many biologists as being the ideal representation of the animal kingdom. A comparison of this artificially arranged Scale of Beings with the stages of individual growth revealed certain similarities between the two series; these were noted by several observers and somewhat rashly generalised into a Law of Parallelism. This law is chiefly associated with the names of Serres and Meckel, but it was suggested and indeed explicitly advanced by earlier workers, as Kielmeyer, Tiedmann,

and Oken, the last of whom could write: "During its development the animal passes through all stages of the animal kingdom."

Meckel himself was not satisfied with the crude enunciation of the so-called law in this fashion, but the first serious criticism was that of von Baer, who pointed out the very obvious facts that a chick, for example, never in its development passes through the stages of a coral, an insect, or a mollusc; and also that many characters appear in the embryonic stages of such a "high" form as a vertebrate which could not possibly have been present in any adult stage of the "lowest" animals—as, for instance, the yolk sac. Further, it is manifest that although a few organs of, say, a developing mammal may recall those of creatures lower in the scale (such as the embryonic gill slits), yet as a whole no stage in the development of the individual can be compared even remotely with a fish, or a bird, or a snail. In other words, the bald pronouncement of Oken is simply not true in fact. Von Baer therefore replaced the Meckel-Serres Law by four laws of development which he formulated as a result of his studies in embryology. These are as follows:

(1) The general characters of the group to which the embryo belongs appear in development earlier than the special characters. (A dog in ontogeny is a vertebrate before it is a mammal, and a mammal before it is a carnivore.)

(2) The less general structures are formed after the more general, and so on until the most special appear.

(3) The embryo of any given form, instead of passing through the state of other definite forms, on the contrary separates itself from them.

(4) Fundamentally, the embryo of a "higher" animal form never resembles the adult of another form, but only its embryo.

In essence, von Baer thus established the principle of deviation: the embryos of two different animals may remain similar for a longer or shorter period, depending on the degree of relationship (that is, similarity of form) between the animals, but sooner or later they diverge and go their own ways: there is no recapitulation, except perhaps recapitulation of stages in the development of individual organs. But it is to be remembered that these laws were enunciated in pre-Darwinian days, and von Baer intended to assert only that there was no recapitulation of stages represented by contemporaneous animals. Although not unaware of the evolutionary concept and its implications, neither von Baer nor Meckel before him made any appeal to palaeontological evidence in the statement of his views.

Although Haeckel is generally credited with the establishment of the Recapitulation Theory in the evolutionary sense, he was, as Russell (1916, p. 252)[1] has pointed out, anticipated in all essentials by Müller, who not merely transferred the Parallelism of Meckel from the Scale of Beings to the Ancestry, but also suggested modes by which ontogeny may diverge from phylogeny by the phenomena that are now known as *caenogenesis* (see p. 110) and *lipopalingenesis* (see p. 115). Indeed, it may be said that all Haeckel did was to give the hypothesis more precise and technical formulation and to elevate it to the rank of a law—the Biogenetic Law. Haeckel

[1] I take this opportunity of acknowledging the great debt I owe to Russell's work for information relating to the history of the early opinions concerning animal development.

added an air of verisimilitude to his doctrine by appealing to the fossil evidence for support, but very largely he ignored the detailed facts and merely referred to the general increase in complexity observable in, say, the vertebrates as they are traced through the geological series. In fact, so convinced was he of the truth of his law, that in Platonic fashion he largely dispensed with such adventitious aids, and formulated phylogenies based solely on the ontogenetic stages of the recent animals, with somewhat unfortunate results. In its initial conception, the Biogenetic Law differed from the Law of Parallelism in little other than its extrapolation in time, to conform with the Darwinian Hypothesis. Nevertheless, however crude may have been his early notions, Haeckel did not fall into all the errors of the transcendentalists, and he was perfectly well aware of the grounds upon which von Baer adversely criticised the Meckel-Serres Law, and of the divergences that may occur between ontogeny and phylogeny. He followed Müller in distinguishing between ontogenetic processes and structures that have phyletic significance, and those that are adaptational to the needs of the larval forms: even he would not have contended that an early ancestor of the dogfish swam in the primeval seas with a food bag suspended from its stomach. Those features which recapitulate ancestral stages he called *palingenetic*, those which are secondarily inserted he called *caenogenetic*. He further recognised that ontogeny may depart from phylogeny by palingenetic characters or processes being disturbed in their relative order of appearance, an instance being the precocious formation of the mammalian heart. To this phenomenon he applied the term *heterochrony*. But it must be again emphasised that these were conceptual phenomena, proposed to conform with an idealistic morphology and an idealistic phylogeny: in no single instance did Haeckel appeal to any established palaeontological succession for confirmation.

It is unnecessary to go into minute detail concerning the chief objections to the Biogenetic Law that were subsequently advanced, mainly by embryologists and comparative anatomists—of whom, however, Oppel at least was well acquainted with the facts of palaeontology. In essence they resolved themselves into an emphasis on the facts of caenogenesis, and on the almost universal occurrence of heterochrony. Thus Mehnert in 1897 wrote: "The assertion is still true that individual organogenesis is exclusively dependent on phylogeny. But we must not expect to find that all the stages in the development of separate organs, which coexisted in any member of the phylogenetic series, appear at the same time in the individual ontogeny of the descendants, because each organ possesses its own specific rate of development."

On other grounds, Giard, His, and Sedgwick used the phenomenon of *poecilogeny* (or embryonic divergence between species which in the adult stages are closely similar, or even inseparable) as the basis for adverse criticism. Following a similar line of argument still further, Hertwig contended that there is as great a real, though perhaps not an apparent, difference between the egg cells of species as between the adults: that a fertilised ovum of a vertebrate is not in the least comparable in essence with the cell of a protozoan, for instance, and the assertion that because it starts life as a unicellular zygote a vertebrate is descended, however remotely, from the

Protozoa, is not a valid conclusion—an opinion with which the Mendelians will agree.

In fact it may be said that at the close of last century, the Biogenetic Law had fallen into complete disfavour, if not disrepute, with the neontologists—a position that is maintained at the present time by such workers as Garstang and de Beer.

Nevertheless, in another field, Hyatt, Cope, Waagen and others were building phylogenetic series from the fossil evidence. And although some of these have scarcely stood the test of time, yet the essential methods of these workers have been continued and developed by such people as Bather, Buckman, Cumings, Grabau, Lang, Swinnerton (1923, pp. 188, 319; 1932, p. 333), and Trueman (Trueman and Williams, 1925, p. 701), who have subscribed to a Recapitulation Theory in which caenogenesis and the effects of acceleration and heterochrony play a modifying part. The purpose of this article is to examine some of the facts upon which these conclusions are based: it is thus complementary to, though more detailed in its examples than, similar attempts by Bather (1893) and Cumings (1909).

III. THE NATURE OF THE FOSSIL EVIDENCE.

In order to determine whether, and to what extent, ontogeny recapitulates phylogeny, it is necessary to be acquainted with both the ontogeny and the phylogeny—a condition that is frequently overlooked, especially by neontologists. This is usually a very difficult matter. Among the cephalopods and gastropods, there is perhaps in many instances an almost complete retention without modification of the skeletal ontogeny in the adult state, while many corals also are little modified. The lamellibranchs and the brachiopods retain most of their *nepionic* (infantile) and *neanic* (youthful) characters on their outer shell surface, though internally there are usually more or less profound changes, in the dentition of the molluscs, in the brachidia and cardinalia of the brachiopods, which are not preserved, or even indicated, in the adult. In most other groups, however, there is scarcely a vestige of earlier growth stages in the *ephebic* (adult) skeleton. The Crustacea, by the periodic ecdysis of their external carapace, effectively destroy, or at least lose, the earlier records. The echinoderms, constantly modifying their test by resorption and intussusception, leave few traces of a diligent or misguided youth. While in the vertebrates, the bones, unlike the hard cases of the invertebrates, grow with the surrounding flesh, and are as completely modified. Ontogeny in such cases is only to be interpreted in terms of a graded series of individuals, which, living contemporaneously, show a gradual increase in size and complexity from an apparent nepionic or neanic stage to an apparent ephebic stage. The method is well illustrated by the determination of trilobite ontogenies (see, for example, Raw, 1925; Stubblefield, 1926). Conditions of preservation, different bionomic relationships of young and adult forms, the accident of discovery, and other factors militate against such a series ever being found, and as a fact they are extremely rare. There is, further, the interpretative difficulty, as to whether a given specimen is truly a young stage of a particular species. Thus while Raymond (1914, p. 228) traces the

ontogeny of the Cambrian trilobite *Paradoxides rugulosus* Corda through the following stages:

$$Hydrocephalus\ saturnoides\ \text{Barrande}$$
$$\downarrow$$
$$Paradoxides\ orphanus\ \text{Barrande}$$
$$\downarrow$$
$$Paradoxides\ pusillus\ \text{Barrande}$$
$$\downarrow$$
$$Paradoxides\ rugulosus,$$
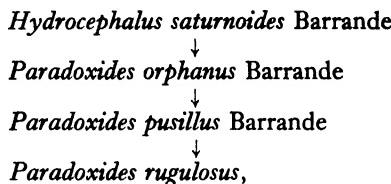
Raw (1925, p. 273), on the other hand, considers the ontogenetic stages of *Hydrocephalus saturnoides* and *Paradoxides orphanus* to lead to the adult *P. bohemicus* Boeck. A similar difference of interpretation occurs concerning the adult form of the *Hydrocephalus carens* Barrande → *Paradoxides inflatus* Corda line of development, Raymond doubtfully considering it to be *P. bohemicus*, whereas Raw favours *P. spinosa* Boeck. It is consequently difficult to be certain of the ontogenies in such cases, despite the most favourable circumstances of preservation.

The phylogenies of most groups are equally dubious. In determining ancestry, the only truly unequivocal method is one of directly observing generation succeed generation until some measurable amount of evolution has occurred, when there can be no doubt as to the progenitors of any particular form. That method failing, as in the vast majority of cases it must fail, succumbing to the brevity of human life, the only other is the discovery of a series of adults morphologically graded in time. The more nearly contemporaneous any two forms, the more nearly should they approach in structural detail, if the series be truly phyletic. The method is thus dependent upon

(*a*) a time sequence, which, in geological terms, means a stratal or zonal sequence,

(*b*) morphological comparison.

The difficulties of stratal correlation over any extensive area are well known, even amongst such exceptionally fossiliferous rocks as the Jurassics. And, inherently and fundamentally, chronological correlation is based upon the evolution of the fossil animals present in the zones—that is, upon the changes that occur in the phylogenies of those animals, which, *ex hypothesi*, are not determined. When therefore the faunal series is not wholly present in a group of deposits confined to a very limited area, the gaps following on migration or stratal failure have to be bridged by evidence obtained elsewhere, in which case the time sequence will lose in precision.

Even so, the chronology of most of the geological systems is now fairly well established, if not in detail, at least on broad lines, and greater liability of error is to be found in the necessity for morphological comparison. This greatly depends on the personal factor, and the divergences which may exist between individual opinions are of the type illustrated above for trilobite ontogenies. To a very considerable degree, much depends upon the initial conception as to the direction of evolution in the groups, as is illustrated, for example, by the different hypotheses of ammonite phylogeny propounded by Buckman (1894, p. 360) on the one hand, who considered that many Mesozoic ammonite families are descended from a *Cymbites-*

like ancestral radicle, and by Spath (1924, p. 189), on the other, who maintains that they are the offshoots of the "fundamental root stocks of Phylloceratidae and Lytoceratidae." Even when observers are less predisposed, the prevalence of parallel evolution and *homoeomorphic*[1] convergence is ever likely to confuse the issue and deceive the interpreter, as is well instanced by theories concerning the evolution of the elephants and of the horses.

In the examples of evolution to be described, the ontogenies of the fossils are as complete and unquestionable as may be expected amongst extinct forms, while in most cases the chronological sequence is straightforward and does not depend upon debatable correlation, and theoretical morphological comparisons are reduced to a minimum. In the last resort, however, it is possible to deny the suggested phylogenies: they are not absolutely established, nor ever can be.

IV. ZAPHRENTOID CORALS.

In the Lower Carboniferous and Millstone Grit rocks of the Northern Province of Britain, there are preserved species of the gens of *Zaphrentis delanouei* Edwards and Haime, whose evolution has been described in detail by Carruthers (1910). They present an almost unbroken succession in time, and at any particular horizon individuals are fairly common. Their evolution does not result in any profound change of structure, and the specimens from any one horizon show only minor differences from those in the next below: there is consequently little possibility of confusion with heterogenetic homoeomorphs of other gentes, which is further removed by a study of the variation at any one horizon. The group is thus eminently suitable for a study of phylogeny.

The typical member of the series, *Zaphrentis delanouei*, occurs in force in the lowest strata concerned—the Cementstones. It is a primitive simple conical or cornute rugose coral, of which a transverse section shows the internal structure to be composed of a number of radially disposed septa that meet in a thick central column; the septa are interrupted by the development of a cardinal fossula which is expanded medially. Associated with the true *Z. delanouei* is a minority of individuals of *Z. parallela* Carruthers, which differs from the former species in its cardinal fossula being parallel-sided and losing its medial expansion. There are also present rare specimens of *Z. constricta* Carruthers, in which reduction of the cardinal fossula has proceeded further, so that its medial termination is clearly demarcated from the remainder by a definite constriction. At the same time, the central column is less massive and the walls of the septa are thinner than in either of the former species, in which features *Z. delanouei* is the more primitive. At the top of the Fell Sandstone, and in the succeeding Lower Limestone Group, the characteristic form is that of *Z. constricta*, but a small proportion of specimens of *Z. parallela*, and rare individuals of *Z. delanouei* are still present. There is also a new arrival *Z. disjuncta* Carruthers, in the early forms of which (those at the top of the Fell Sandstone) the

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[1] *Homoeomorphy:* The development of similar form in organisms of different ancestry; as, for example, in the cases of the whales and the sea-cows, the bats and the pterodactyls, coat-of-mail shells (*Chiton*) and wood-lice.

central column is much reduced, and the septa partially detached, while in the later forms (of the Upper Bernician) the column is completely absent and the septa do not extend to the centre of the coral and may indeed be themselves reduced to the thinnest vestiges.

The time sequence of forms may be illustrated by the distribution curves of Fig. 1. In the Upper Limestone Group, *Z. disjuncta* becomes the dominant species, while specimens of *Z. constricta* form a notable minority; occasional specimens of *Z. parallela* still occur, but *Z. delanouei* is completely absent. Finally, the Millstone Grit forms are almost wholly composed of an advanced form of *Z. disjuncta*, in which the septa are the merest threads; a small proportion of *Z. constricta* persist, but the earlier species are absent.
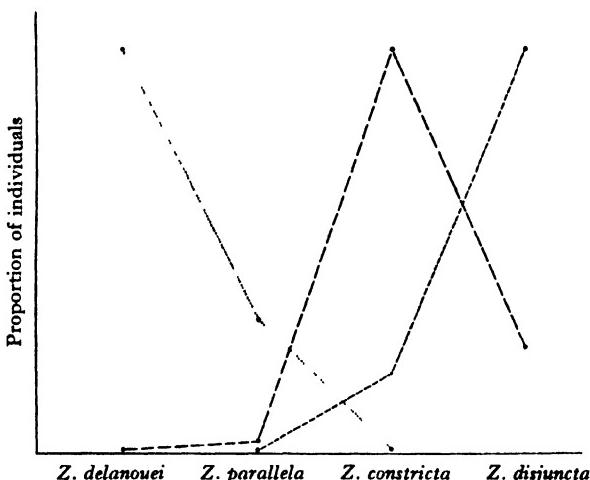
Fig. 1. Variation curves showing the distribution and evolution of the species of the gens of *Zaphrentis delanouei* in the Carboniferous rocks of the North of England. Based on Carruthers. Cementstone Group . . . ., Lower Limestone Group — —, Upper Limestone Group – – –.

There is, then, in this evolving group an excellent example of a lineage that is characterised by three main trends:

(1) Modification of the cardinal fossula;
(2) Decrease in thickness and extent of the septa; with which is associated
(3) Decrease in thickness of the central column.

The evolutionary changes are characterised throughout by a complete absence of saltation, and although in the above description, which follows that of Carruthers, it has been convenient to refer the forms to arbitrarily separated "species," it will be appreciated that in fact there is a continuous series of transitional forms between each, both in space and in time. That is, there is no discontinuity in either the contemporaneous variation or in the mutation[1]. This is, therefore, as completely perfect

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[1] In Waagen's sense. So throughout the article, except in quotations.

an example of the evolution of a gens as one may hope to find amongst invertebrate fossils, the sequence being:

Zaphrentis delanouei
↓
Zaphrentis parallela
↓
Zaphrentis constricta
↓
Zaphrentis disjuncta.
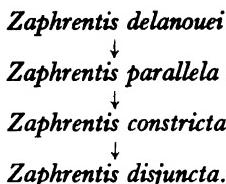
In ontogeny, *Zaphrentis parallela* develops through a neanic stage in which the cardinal fossula is expanded towards the massive central column—that is, the appearance of its youth is similar to that of the adult *Z. delanouei.* In like manner, *Z. constricta* passes through a neanic stage in which the fossula is parallel-sided, and the septa and central column are more massive than in the adult; in this species, however, the *delanouei* stage is not present even in the earliest formed parts of the skeleton. A few of the more primitive forms of *Z. disjuncta*, those from the Lower Limestone Group, begin their life history with a parallel-sided cardinal fossula, the septa meeting in a well-developed central column; this is followed at a later stage by a constriction of the fossula. In the majority, however, and in all specimens from high horizons, the *constricta* stage is the first to appear, and is rapidly followed by a retreat of the septa and a disappearance of the central column, leading to the typical ephebic form.

The ontogenies may be tabulated as follows:

Species (ephebic stages)	*Zaphrentis parallela*	*Zaphrentis constricta*	Early *Zaphrentis disjuncta*	Late *Zaphrentis disjuncta*
		—	constricta	constricta
Ontogenetic stages	—	parallela	parallela	—
	delanouei	—	—	—

If the phylogeny be unquestioned, the conclusions to be drawn are obvious:

(1) The ontogeny of each species recapitulates its phylogeny. The young stage of each species is more similar to the adult stage than it is to the young stage of its immediate ancestor.

(2) There is a condensed development, a "pressing back," a *tachygenesis* of the ancestral stages in the ontogeny of the later descendants.

(3) This tachygenesis may proceed so far as to repress completely, to push off the end as it were, the earlier stages (such as the *delanouei* stage in the ontogeny of *Zaphrentis constricta*, the *parallela* stage in the ontogeny of *Z. disjuncta*), which are skipped in later descendants. That is, *lipopalingenesis* occurs.

A very similar instance is that of certain Silurian zaphrentoid corals described by Ryder (1926). The three genera *Pycnactis*, *Mesactis*[1], and *Phaulactis*

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[1] Lang and Smith (1927, p. 471) have referred the genotype of *Mesactis* (*Mesactis glevensis* Ryder) to *Phaulactis*, believing the differences between the genera to be insufficient to warrant the elevation of the former to generic rank. This suppression of *Mesactis* does not affect the argument, however.

show a seriation in their adult features which is marked by the following trends:

(1) Reduction in the amount of stereoplasm, as measured by the thickness of the septa.

(2) Shortening of the septa, both by
  (a) a retreat from the centre (amplexoid trend[1]), and by
  (b) a retreat from the epitheca (lonsdaleoid trend).

(3) An increase in the number of septa, and a tendency to lose the initial bilateral symmetry as a quasi-radial symmetry is developed (cyathophylloid trend).

(4) The introduction and widening of the epithecal zone of dissepiments (cystiphylloid trend).

Pycnactis exhibits an unmodified zaphrentoid stage, in which bilateral symmetry is well developed. The septa are excessively thick, and, except in parephebic and gerontic stages, form an almost solid mass of stereoplasm, though the divisions between the septa remain distinct; there is no development of dissepimental tissue. In *Mesactis*, the early stages of all four trends may be observed. Though the septa remain thick and in lateral contact over the greater portion of their length, the central portion of the coral is devoid of skeleton, while the retreat of the septa has commenced and is accompanied by the formation of a narrow outer ring of dissepiments. In *Phaulactis*, the septa are exceedingly thin, compared with those of the other genera; they are greatly increased in number and assume a quasiradial symmetry; the median cavity is increased in size; and the outer dissepimental zone occupies about two-thirds of the corallite. The adult seriation may be expressed thus:

Pycnactis → *Mesactis* → *Phaulactis*.

The ontogeny of *Mesactis* shows a neanic stage in which the septa are excessively thick and in lateral contact along their margins; they meet at the centre of the corallite, where there is little or no suggestion of the amplexoid trend; they are thickest where they join the epitheca—the lonsdaleoid trend is not begun; and there is no outer dissepimental zone. The stage is one that, were it not followed by another in which the four trends are more or less incipiently developed, would result in the form being included within the genus *Pycnactis* without question. Similarly, the nepionic stages of *Phaulactis* show the typical zaphrentoid development of the stout septa meeting at the centre, with no epithecal dissepiments; while in the neanic stages the trends begin to operate and the typical *Mesactis* stage is present, leading to the typical ephebic *Phaulactis*. In other words, *Mesactis* in its ontogeny passes through a *Pycnactis* stage, while *Phaulactis* in its ontogeny passes through an abbreviated *Pycnactis* stage and then through a *Mesactis* stage; and the youthful stages of *Phaulactis* are more similar to the adult *Mesactis* than they are to the young *Mesactis*, which again is like the adult (and incidentally, but to a less degree, the youthful) *Pycnactis*.

Again it is difficult to avoid the conclusion that the ontogeny of *Phaulactis* is a condensed representation of ancestral adult stages.

[1] For definitions of these terms, see Lang (1923).

There are other comparable cases amongst rugose corals, which might be adduced as evidence of the same kind. Such are, for example, the probable development of the Lower Carboniferous genera *Siphonophyllia* and *Caninia* out of *Zaphrentis*, in which a series of form changes occurs directly parallel with that of the *Pycnactis* group (see, for example, Lang, 1923, p. 129; O'Connell, 1914); the development of *Aulophyllum* through a *Rhodophyllum* stage from "*Clisiophyllum*" (Smith, 1913; Lang, 1923, p. 128); and possibly the development of thamnastraeoid species of *Orionastraea* from astraeoid species of that genus, which again are descended from species of *Lithostrotion* (Smith, 1917; Hudson, 1929). These are not used as examples in this paper, however, as the precise phylogenies of the various groups are open to more than one interpretation (which, of course, is the essential difficulty of morphological comparison), so that the corresponding phylogenetic equivalent of any particular ontogenetic stage cannot be determined unequivocally. Nevertheless, the kind of evidence they offer is exactly of the type described above for the *Zaphrentis delanouei* and *Pycnactis* groups. But while in the latter groups the correspondence of phylogeny with ontogeny may be demonstrated, in the former it is merely probable.

V. LIASSIC OYSTERS.

The evolution of the genus *Gryphaea* presents details as regards both phylogeny and ontogeny that considerably illumine the present inquiry. The forms have undergone minute analysis at the hands of Trueman (1922 *a*, 1930; see also Swinnerton, 1929, p. 158; 1932, p. 322).

The lowest strata of the Lower Lias are characterised by the presence of an ostraeiform lamellibranch, *Ostraea liassica* Strickland, which is not greatly dissimilar from the present-day *O. edulis*. It is cemented to a support, sometimes the tests of other shell-fish, by the whole surface, or nearly the whole surface, of the left valve. In succeeding zones of the Lower Lias, oysters appear which show a gradual decrease in the area of attachment, which decrease is accompanied by an incurving of the umbo of the left valve, and the development of the gryphaeate form in the shape of the shell. Ultimately the incurvature proceeds to such a degree that the umbonal portion of the left valve presses upon the lid-like right valve, after which no further progress is possible, and the lineage becomes extinct (Fig. 2).

The phyletic stages along this line of evolution are represented by the following species:

<div align="center">

Ostraea liassica
↓
Ostraea irregularis Munster
↓
Gryphaea dumortieri Joly
↓
Gryphaea obliquata Sowerby
↓
Gryphaea incurva Sowerby.

</div>
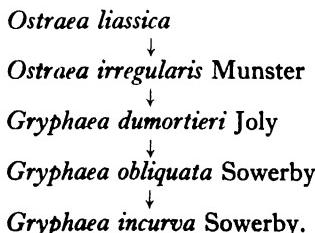
The development is absolutely transitional, so that not only is the distinction between two consecutive species wholly arbitrary, but the separation of *Gryphaea* from *Ostraea* is itself equally unreal (Trueman, 1924, p. 357). Furthermore, this transition occurs not only in time, but also at any particular horizon, where the group concerned obeys the ordinary laws of variation and falls along a normal variation curve when the degree of coiling and the relative size of the area of attachment (which are obviously correlated) undergo measurement. There is thus as complete an elimination as is possible with fossil forms of the liability of error resulting from parallel development and homoeomorphic resemblance.

Again, the time sequence is not dependent upon correlation at a distance, for all the forms concerned are present in the Lower Lias of the South Glamorgan coast, where the stratal sequence can be determined straightforwardly merely by noting the superposition of the beds.

The phylogeny propounded by Trueman cannot therefore be impeached, and the example is as unquestioned as that of the Zaphrentids described by Carruthers. It may be illustrated in the same way by variation curves showing a time sequence, as in Fig. 3.

The ratio of length of area of attachment to total length of shell is approximately as follows:

		%
Ostraea liassica ...	...	90
Ostraea irregularis	...	50
Gryphaea dumortieri	...	30
Gryphaea obliquata	...	10
Gryphaea incurva	...	4
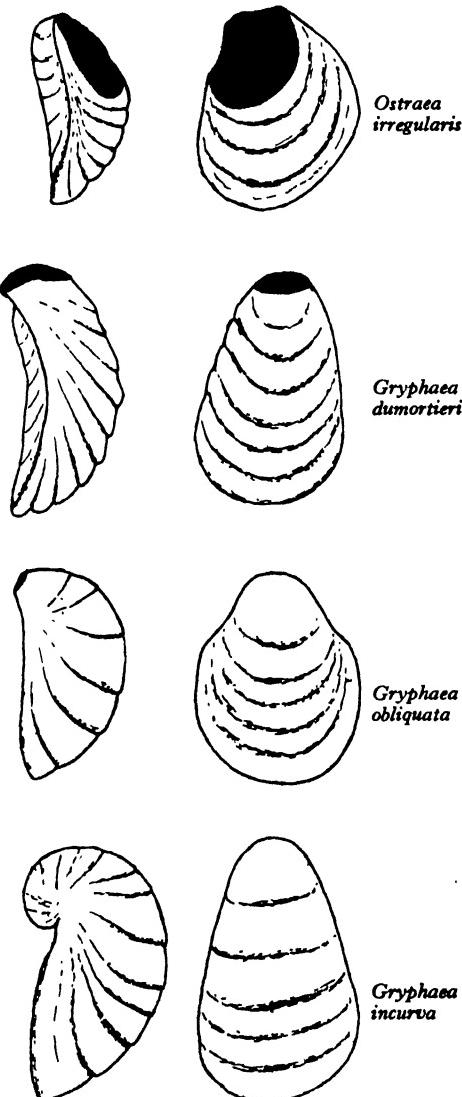
Fig. 2. Form changes in the shape of the shell in members of the gens of *Gryphaea incurva*. After Trueman.

though again it will be appreciated that these are arbitrary distinctions.

During ontogeny, the shell remains attached throughout the nepionic stages of all forms, but sooner or later, according to the degree of incurvature of the adult

shell, it breaks away from the support and leads a free life. The stage in the life history when the animal ceases to increase the area of attachment is of course indicated by the relative size of that area, and in this feature the ontogeny is completely retained in the adult. It will be observed that up to the time when there is no further increase in size of the area of attachment, the left valve remains virtually wholly attached. The ontogenies of the various species may then be summarised as follows:

Length of area of attachment as a percentage of the length of shell	Stage of growth as a percentage of the length of the adult shell in			
	Ostraea irregularis	*Gryphaea dumortieri*	*Gryphaea obliquata*	*Gryphaea incurva*
90 % (≡*Ostraea liassica*)	60	33	11	5
50 % (≡*Ostraea irregularis*)	100	60	20	8
30 % (≡*Gryphaea dumortieri*)	—	100	33	13
10 % (≡*Gryphaea obliquata*)	—	—	100	40
4 % (≡*Gryphaea incurva*)	—	—	—	100

Again the facts point the conclusion. *Gryphaea incurva*, when it is one-twenty-fifth grown, is in the stage of *G. obliquata* one-tenth grown, or of *G. dumortieri* one-third grown, or of an *Ostraea irregularis* three-fifths grown, or of an adult *O. liassica*
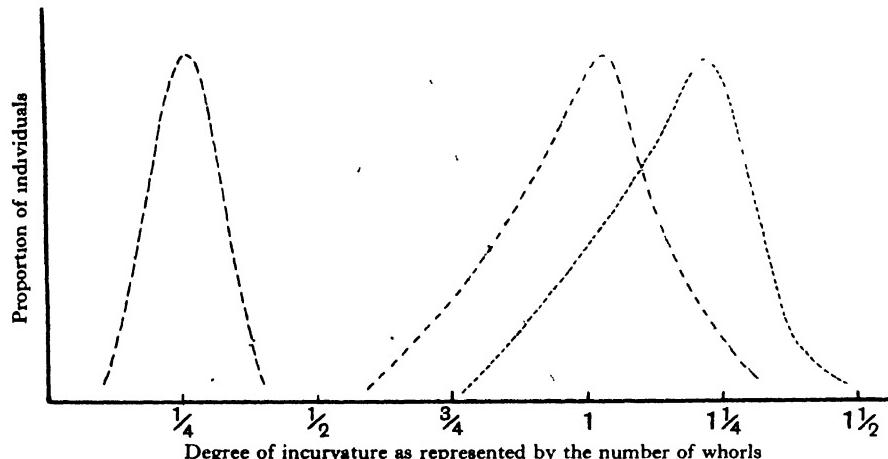
Fig. 3. Variation curves showing the distribution and evolution of species of the gens of *Gryphaea incurva* in the Lower Lias. After Trueman. Lower *angulata* zone – – –, *vermiceras* zone . . . ., *rotiformis* zone – · – · –, *gmuendense* zone - - - -.

(Fig. 4). Or, to put it another way, a very youthful *Gryphaea obliquata*, but one-fifth adult size, is more similar to the adult *Ostraea irregularis* than to the young *O. irregularis*. It would seem that there is no avoidance of the inference that the later forms in their ontogeny pass through the main stages of their phylogeny; and that in the ontogeny of such a form as *Gryphaea obliquata* there is a condensation, a tachygenesis.

Comparable gryphaeoid trends are to be observed in other oyster stocks, re-

sulting in end-forms very similar to *Gryphaea incurva*. Such, for example, is "*Gryphaea*" *sublobata* of the Gryphite Grit (Inferior Oolite), while "*Gryphaea*" *vesicularis* of the Chalk is similar to *Gryphaea obliquata*. In the cases of these later forms, no detailed phylogeny has been determined such as Trueman has established in the Liassic oysters. But with the analogy of the latter, the presumption that they evolved from ostraeiform ancestors by incurvature of the left valve is scarcely to be
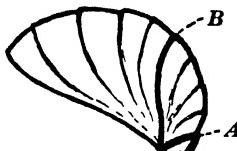
Fig. 4. Ontogeny of shell shape in *Gryphaea* aff. *obliquata*, showing the different morphogenetic stages passed through. *A*, the *Ostraea irregularis* stage; *B*, the *Gryphaea* aff. *dumortieri* stage. After Swinnerton.

denied. Yet without such analogy, an examination of their ontogenies would imply an identical phylogeny—provided the essence of the Biogenetic Law be true. That is, the application of a theory of palingenesis, and of evolutionary theory on a morphological foundation, lead in this instance to the same result.

VI. AMMONITES.

Garstang (1922, p. 93), in his indictment of the Biogenetic Law, confesses that though frequently tempted "to attack a theory which had led so many into blind alleys...always Hyatt's Ammonites recurred to present an unanswered, and seemingly unanswerable case for Haeckelian recapitulation." A discussion of Hyatt's ammonites, therefore, can scarcely be left out of any consideration of the problem, though it must be confessed that they are rather more susceptible to criticism than perhaps Garstang is aware. They suffer from two bad faults: in the first place, there are too many of them, and in the very mixed crowd it is most difficult to say of this one that yonder is his father—so often is it a case of mistaken identity; in the second place, they were a restless lot, and the grandparent of one buried in the muds of Dorset may have known the seas of Jura or the cliffs of the Western Isles. The phyletic sequence is, in other words, obscured by homoeomorphy and migration.

The consideration of ammonite phylogeny is mainly concerned with shell shape and ornamentation. As to the latter, Garstang has drawn a comparison between the larval stages of the prosobranch gastropod *Lamellaria* and the features often exhibited by ammonites. *Lamellaria* passes through a number of stages, including tuberculate, spinose, and costate stages, which are wholly larval, and which Garstang suggests are caenogenetic, and dependent as regards degree of development upon local environmental conditions and the inherent constitution of the animal. He concludes that "if this is so for the 'caenogenetic' larvae of *Lamellaria*, it is not likely to have been very different for the 'palingenetic' stages of Ammonites." In the first place, however, it may be remarked that *Lamellaria* is an extremely aberrant form and differs from nearly all other gastropods in this development, which is by

no means explained as to the separation of the shelly layers, the relations with the soft parts, or the constant resorption of the earlier formed portions of the larval shell. An analogy of the development of normal gastropods (such as members of the Cerithiidae[1]) with that of ammonites would be more pertinent. Secondly, all known ammonites possess a nepionic stage which is totally unornamented, and however much "pressing back" may occur (if it does occur) in the ontogeny of later members of phylogenetic series, the ornamented stage is never telescoped into the initial whorls. That is, even if the development of ornament in the larva of *Lamellaria* is a caenogenetic feature in Garstang's sense, and even if it foreshadows a future phylogeny in which adults will be so ornamented (which is very far from being proved, or even from being probable), and even if the analogy of this larval gastropod shell with the shell of ammonites is valid, yet in fact no ammonite "larva" ever possessed any sort of ornament on its shell which could have been so impressed upon subsequent phylogeny. In all ornamented ammonites, the incipient stages of the ornamentation first appear in post-nepionic stages.

But let the ammonites speak for themselves.

The possible changes in shell shape and in ornamentation being more or less limited, and there being such an abundance of ammonites, it is necessary to have some classificatory anchor other than superficial resemblance, if the shoals of homoeomorphy are to be avoided. This is provided by the septal suture, which, being very considerably complicated, is scarcely likely, as a mere matter of probability, to be closely similar in two independent evolutionary series. In the ensuing remarks, it is assumed that a use of the suture in this way is justified, if only because in actual practice the application of such a classification is found to unite ammonites into obvious family groups, and to separate groups chronologically as well as spatially. The assumption may be denied, however, when the argument and the conclusions become uncertain, though not invalid.

The upper part of the Lower Lias is the age of the Liparoceratidae (Trueman, 1919). These ammonites are represented in the lowest zones by forms which are evolute and slender in whorl shape and which possess an ornament of coarse ribs that cross the venter (a capricorn ornament). In several parallel series (not necessarily lineages), there is a morphogeny in the group which is characterised by the following features:

(1) A change in whorl shape from evolute to globose involute.

(2) A development of first a paired bituberculate stage, then an unpaired bituberculate stage, succeeding the capricorn stage.

That is, there is a change from an early capricorn serpenticone stage through various intermediate stages to a bituberculate sphaerocone stage.

The ontogeny both of whorl shape and of ornament is of course retained *in toto* to the adult stage.

The ontogeny of the ornament is represented diagrammatically in Fig. 5, which is modified after Trueman. The arrows indicate the chronological sequence of forms.

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[1] See, for example, McDonald and Trueman (1921).

It should be remembered that at the same time there is a development of a swollen whorl. In *A*, the ontogeny is the simple one of smooth initial whorls being followed by the capricorn ribs. In *B*, the capricorn stage is shortened, and is followed in the adult by a stage with paired tubercles. "Owing to the method of origin of the tubercles as points on the ribs, they are first necessarily arranged in pairs. When the whorl becomes swollen, however, the line of outer tubercles is much longer than the line of inner tubercles," so that the number of outer tubercles progressively increases relative to the number of inner tubercles. This is well illustrated in *C*, where the capricorn stage exists for only a portion of the whorl. In the same way, the capricorn portion of *C* is evolute, the paired tuberculate whorls are stout, the unpaired tuberculate whorls are swollen and involute. It is a simple statement of fact to say that the ontogeny of *C* is a recapitulation of its phylogeny (if the seriation of transitional forms be accepted as equivalent to the phylogeny), though each ontogenetic stage is necessarily abbreviated when compared with the corresponding
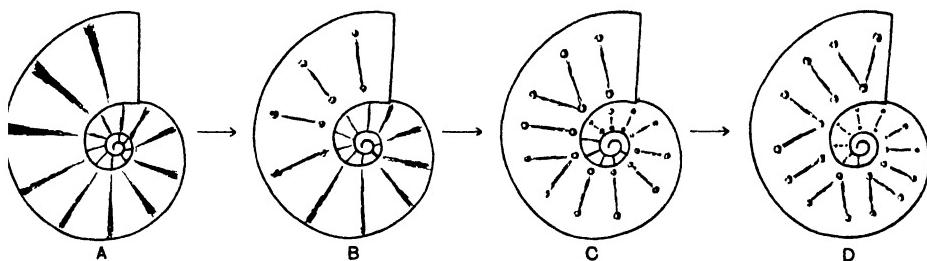
Fig. 5. Diagrammatic representation of the ontogeny and morphogeny of ornament in members of the Liparoceratidae. After Trueman.

ancestral adult (as must always be the case unless there is also an indefinitely unlimited increase in size as evolution proceeds). The accuracy of this suggested phylogeny is supported by chronological sequence, morphogenetic development, and uniformity of sutural plan, though it is not contended that clear-cut lineages may be determined. As in the case of *Gryphaea obliquata*, therefore, an examination of the ontogeny of the *C*-stage in this line of evolution would give an accurate synopsis of the phylogenetic series of adult ancestors.

But there is a further stage, *D*, which differs only slightly in the adult from *C*— it is an unpaired bituberculate sphaerocone. In its ontogeny, however, tachygenesis has proceeded further, and the capricorn stage, and with it the evolute whorl, are completely absent, so that the animal starts life with the usual depressed protoconch and involute nepionic whorls, and immediately proceeds to add to them further involute whorls with tuberculate ornament. Lipopalingenesis of the evolute capricorn stage has occurred, and there is no evidence in ontogeny of a prominent stage in phylogeny. The case is not very different from that of the *Zaphrentis delanouei* group above described, in which the *delanouei* and *parallela* stages are omitted from the ontogenies of the later forms. There is one notable difference, however, for in the Zaphrentids the earlier stages are passed over, as it were, at the beginning of

growth, the animal starting at a late (skeletal) stage (as the *constricta* stage in *Zaphrentis disjuncta*). In the Liparoceratids, on the other hand, the earliest nepionic whorls of all the forms are virtually identical, and when the capricorn stage is skipped, it is not passed over at the commencement of ontogeny, but is squeezed out between the initial smooth depressed whorls and the ever-encroaching tuberculate depressed whorls. But tachygenesis of the latter is not the sole reason for the elimination of the capricorn stage: the depressed nepionic whorls of *D* persist to a greater diameter than in the original *A*, and the skipping of the slender-whorled costate stage "is accomplished not simply by the acceleration of the stout form of whorl but partly also by an apparent retardation of the early characters" (Trueman, 1922 *b*, p. 143). To this phenomenon, Grabau (1910, p. 54) has applied the term *bradygenesis*; it is a particular instance of the more general heterochrony.

With these examples established as certainly as may be, it is possible to consider other ammonite groups in which migration and parallel development play a more disconcerting part.

The upper part of the Blue Lias (the Lymian) is characterised by a group of ammonites, the Arietidae, in which the general shape of the earlier forms is polygyral serpenticone, with keels more or less sharply developed along the venter; the ornament consists of variously developed usually widely spaced ribs which are not continuous across the whorl. The earlier forms are slightly compressed, and the keel is not sharply delimited from the flanks. But in the forms occurring in the mid-deposits of the Lymian, the whorls tend to become laterally inflated and to assume a coronate outline, typified by *Coroniceras* itself, in which the keel is bounded by two lateral furrows. Still later in time, *Paracoroniceras* appears. It is obviously a member of the same group, not differing very greatly in form, and possessing essentially the same plan of suture. But owing to the fact that many of the multitude of arietidan forms bear a greater or less resemblance to each other, it is difficult to determine the precise line of ancestors along which *Paracoroniceras* evolved. That is, the phylogeny of the form is not established with certainty. Nevertheless, it is manifest that whichever particular species gave rise to *Paracoroniceras gmuendense*, that particular species must have been evolute stout-whorled costate carinate-bisulcate, for there was no other type from which it could have evolved—or at least, no other known. *Paracoroniceras*, however, differs from the *Coroniceras* group in being relatively involute in the ephebic stage, with a compressed whorl, and with costate ornament only feebly developed. It thus displays an evolution in change of whorl shape (which is more sharply keeled and suboxyconic) and in a *catagenesis* (decline) of ornament. But the same changes are manifest in the ontogeny of *Paracoroniceras*, which, after the initial smooth depressed nepionic whorls, passes through an evolute coronate crassicostate neanic stage before attaining the compressed whorls of the adult. If the assumed phylogeny, or at least morphogeny, be true in essentials, then it is reproduced in the ontogeny.

A further step may be permitted. At the close of the Lymian, the *Coroniceras* group very largely disappeared from the north-western European province, and throughout the Mercian they are virtually unrepresented. But in the uppermost

beds of the succeeding Deiran stage, and in the Raasayan stage, there appeared a self-contained group of forms which in their morphology present many analogies with the earlier arietids. In view of this gap in the biological succession, occasioned by migration, their descent from the latter can, however, only be premised; it may at least be said that if they are descended from any known forms, they are most probably descended from the *Coroniceras* group—a probability on which most observers are agreed[1]. They carry the trends of *Paracoroniceras* to further stages, and are extremely involute, compressed, sharply keeled (oxyconic), and almost completely smooth in the adult stage. The ontogenies of some of the forms have been studied in detail (George, 1930), and though there are considerable minor differences, in essence they go further than *Paracoroniceras* in the tachygenesis of the evolute coronate stage, which may be so accelerated as to be skipped altogether from ontogeny, when there is a direct development from the involute depressed nepionic whorls to the involute compressed ephebic whorls—admirably exemplified in

Coronate   Paracoronate   Oxyconic
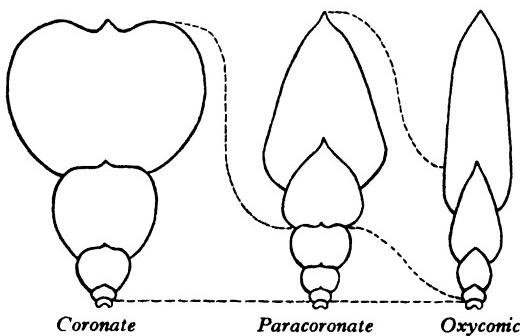
Fig. 6. Transverse sections through the shells of arietid ammonites from the Lower Lias, showing the ontogeny and morphogeny of whorl shape, with tachygenesis and lipopalingenesis occurring in the oxyconic forms. After Swinnerton and George.

Oxynoticeras oxynotum (Quenstedt) itself (Fig. 6, with which compare Swinnerton, 1923, p. 206.) Even so, the ornament of *Oxynoticeras* is not quite eliminated, and the smooth or almost smooth adult whorls are prefaced in ontogeny by a neanic stage in which relatively coarse ribs are developed. Assuming the suggested phylogeny, therefore, the ontogeny of *Oxynoticeras* is made more direct by the elimination of a palingenetic deviation. The analogy with the Liparoceratidae, in which lipopalingenesis of the capricorn stage removes the palingenetic deviation, is obvious. There is one slight difference, however, in the rates of acceleration: while in the Liparoceratidae the capricorn ornament and evolute whorl shape approximately accompany each other through all the phyletic and ontogenetic stages, in the oxycones, the elimination of the ornament lags behind that of the coronate whorl shape, and the latter is absent in *Oxynoticeras*, for example, while ornament, though feebly developed, remains. To such relative acceleration, Buckman applied the term

[1] Thus Spath (1924, p. 189), who disavows ontogenetic evidence, puts *Oxynoticeras* in the same super-family as *Coroniceras*.

precedentive palingenesis; it is included within Haeckel's heterochrony. Manifestly, it has a notable effect in obscuring the true phylogeny, if an attempt be made to found the latter on ontogeny, but its effect is less disastrous than that of lipo-palingenesis.

With these conclusions, one may well agree with Spath (1926, p. 139), who, on other grounds, remarks "it is clear that a simple application of the biogenetic law... would lead to results as absurd as in the case of a new-born monkey." The application of the law must be tentative, rather than final; it is approximate, rather than exact; it suggests and implies, rather than fulfils. When its implications lead to anomalous chronological results, as apparently is the case with some of the amaltheids, then obviously it must be applied with caution, though even in such cases it may be a valuable aid in the construction of palaeontological theory, as in the conception of homoeomorphy and parallel development. It certainly does not necessarily follow that the law is "discredited": it is merely more complicated than was formerly supposed, and it is the rash application of the law that merits censure.

The instances given above in part answer the criticisms of de Beer (1930, p. 51), who suggests, in contradiction to Lang (1919, p. 50), that the ornament of *Microceras densinodus* may have resulted from deviation, rather than recapitulation. He asks the question, "why should the ribbed shelled stage in the ontogeny of *Microceras densinodus* represent an ancestral *adult* stage? That ancestor may have had a ribbed shelled early ontogenetic stage, but there is no evidence at all as to what its adult state may have been like." One cannot answer for *Microceras densinodus*, of whose phylogeny, though unknown, de Beer is prepared to speculate in such facile fashion, but the case is almost identical with that of Form C in the *Liparoceras* series, of which it is well-nigh certain that the ancestors were first capricorn and then incipiently tuberculate in the adult stage. He further adds that "indeed, in some species of *Psiloceras*, Spath has shown that the ribs make their first appearance in phylogeny in the inner whorls, *i.e.* the early stages of ontogeny." As *Psiloceras* first appears, presumably by migration, in the lowest beds of the Lower Lias, which rest on Rhaetic deposits devoid of ammonites, it may be pertinent to ask where the laevigate ancestors of this ribbed form occur, and by what means the phylogeny was determined if none of the ontogenetic details were taken into consideration. While chronology forbids the hypothesis of a descent of *Psiloceras* from the *Caloceras* group, which is indicated by the ontogeny, yet it is evident that in these Lower Liassic phylloceratoids there were a number of heterochronous parallel series, comparable with those of the Arietidae and the Liparoceratidae, and it may well be that *Psiloceras*, though not descended from *Caloceras* itself, nevertheless had a *Caloceras*-like ancestor. So much, however, is mere surmise, and in this instance migration and homoeomorphy so confuse the problem that the evidence cannot be certainly used one way or the other. All of which may be said without necessarily denying any part played in phylogeny by juvenile interpolations.

VII. GASTROPODS.

The gastropods are important, as Garstang has used them as evidence against Haeckelian doctrine. Of the phyletic significance or otherwise of the veliger larva, which looms large in Garstang's arguments, a palaeontologist can say nothing. It would seem, however, that the phylogeny of the group which he propounds is every whit as speculative and as preconceived as that of any Haeckelian. His analogy of phylogeny with a contemporaneous morphological "systematic sequence" contains in essence the prime falsity of the Meckel-Serres Law. And his desire, "if torsion arose in the first instance by gradual modifications of the adult form," to find "somewhere over the wide earth...a Zygobranch snail with its torsion incomplete" may not be so reasonable as he thinks; Palaeontology has unearthed numerous extinct annectent types without whose discovery the relations between different groups might at the best be but darkly suspected: *Archaeopteryx* will suffice as an example. And if by chance torsion did arise by gradual modifications, it may well be that all the changes to complete torsion had been passed through before the end of dim Cambrian days—before, perhaps, the gastropod ever got its shell. In any case, we shall never know, for the rocks do not preserve the twists of long ago. And one guess is much the same as another.

Whether, however, the torsion occurred by gradual modification or by saltation, whether palingenetically or caenogenetically, it has left one relic which is preserved fossil—the slit or perforation of the Zygobranch shell. Garstang has used the facts concerning the "history and function of the Zygobranchiate slit...to disentangle the elements of truth and error which Haeckel so confused in his 'Biogenetic Law.'" He claims that though *Fissurella* goes through a slotted stage in its development, it does not follow that this *Emarginula*-stage represents an ancestral adult condition, for whereas the adult *Emarginula* possesses not merely a marginal slit but also a long slit band which indicates the migration of the slit during ontogeny, the slit only is present in the young *Fissurella* and becomes sealed marginally without any development of a slit band.

Unfortunately, although the forms are abundant throughout the Tertiary, and less commonly in the Mesozoic and Upper Palaeozoic, their phylogenies are wholly unknown, and hypotheses as to ancestry are consequently highly speculative. But, with the analogy of *Gryphaea* and the corals before us, it seems that the case of *Fissurella* is not wholly inexplicable by Haeckelian methods. For the advanced specimens of *Gryphaea incurva* possess the minutest relic of an attached stage, and a study of the ontogeny of that species would of itself provide little indication of an ostraeoid stage in phylogeny. Similarly, the *delanouei* stage is missing from the ontogeny of the later mutations of this zaphrentoid series. These condensations and ultimate deletions of phyletic stages in ontogeny are presumed to have resulted from extreme acceleration leading to lipopalingenesis. Though the apical perforation of *Fissurella* is necessarily retained throughout ontogeny, yet the accompanying slit band seen in other genera, of which no trace is present in *Fissurella*, may have been eliminated from ontogeny by a similar acceleration in phylogeny. That is, although

Fissurella nowadays shows no sign of an *Emarginula* stage in the presence of a slit band, the absence of the band does not prove it is not gradually evolved from ancestors which in the adult stage possessed a marginal slit or a lateral hole. On this Haeckelian hypothesis, the adult seriation runs as follows:

Marginal slit, long slit band (compare *Emarginula*)
↓
Lateral hole, shorter slit band (compare *Rimula*)
↓
Subapical hole, very short slit band
↓
Apical hole, no slit band (*Fissurella*).
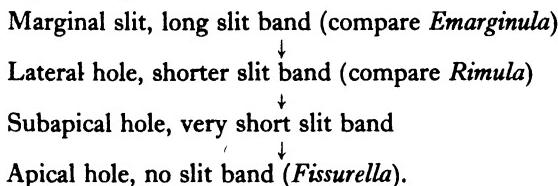
These are, of course, mere surmises, but they demonstrate the possibility of a modified recapitulation even in such an exceptional case as this.

Incidentally, it is fairly certain that *Fissurella* (or *Fissuridea*, its homologue) is evolved from a form with a marginal slit, for it is not known from pre-Carboniferous rocks, during which times all Zygobranchs possessed a marginal slit and a long slit band. The evolution of *Fissurella* from the latter could then proceed either by mutation (the Haeckelian method) or by saltation (Garstang's method). And although we may not be under any "intellectual necessity of concluding that everything must arise late in the life history," yet such a saltation, from marginal slit to apical hole, is one that taxes the imagination of even the most heterodox evolutionist. Presumably the lateral holes of different species of *Rimula*, at various positions between the apex and the margin, are supposed also to have arisen by saltation. If this is so, then the saltation was not larval; it occurred during neanic or orthephebic stages; for the slit in *Rimula* is not closed until such a late period in ontogeny. And a late neanic saltation that persists in phylogeny is a hitherto unknown mode of evolution. It seems, therefore, that a lipopalingenetic hypothesis of the evolution of *Fissurella* is at least as credible as one based on caenogenetic saltation.

VIII. BRACHIOPODS.

Many genera of brachiopods pass through variously ornamented stages in their phylogeny and in their ontogeny of a type directly comparable with those of the ammonites and the gastropods, in which there is generally a progression from smooth through costate to tuberculate (spinous) stages. These need not be considered in detail, as the evidence they present in support of recapitulation is closely comparable with that, for example, of the Liparoceratidae. It will be sufficient to mention the Atrypidae of the Silurian, the brachythyrid spirifers of the Carboniferous, the Tertiary tegulorhynchs. In essence they all illustrate the principle of tachygenesis, and in a few, this results in lipopalingenesis. It may be observed that Garstang's *Lamellaria* can scarcely be compared with the protegulum of brachiopods, on which no ornament is ever seen: indeed, in many brachiopods it may be demonstrated that the spinous stages result from the interaction of transcrescent ornamental features with the enhanced concrescent growth lines (see, for example, C. Leidhold, 1922, Pl. XIII).

More interest attaches to the shell form.

The primitive stage of most brachiopod series is one in which the two valves of the shell are biconvex and meet in a straight anterior margin. From this recti-marginate stage, the shell may become modified by the development (possibly for physiological reasons, resulting in a more efficient mechanical separation of the


R. lobata

R. obtusa

R. mesoloba
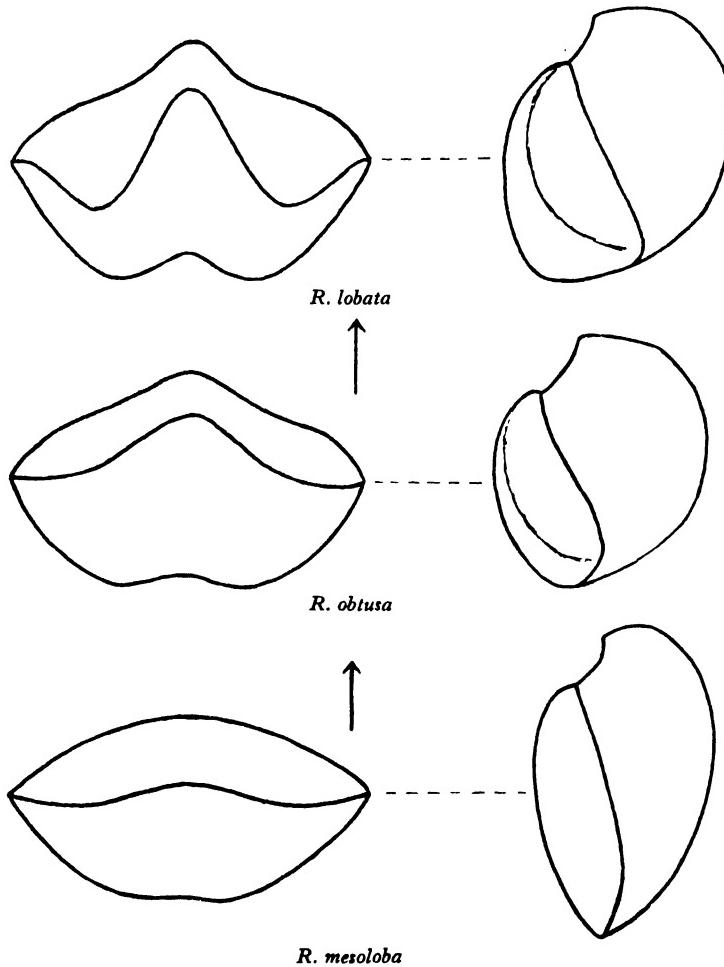
Fig. 7. Ontogeny and morphogeny of the shell form in species of *Reticularia*, showing tachygenesis in the development of the median fold. After George.

inhalant and exhalant food-bearing currents) of a median fold in one valve com-plemented by a corresponding sulcus in the other. This median fold may become enhanced by an increasing steepness of the sides and by the development of lateral furrows, so that in anterior view the margin appears as a sharply plicated **W**. This seriation is well exemplified by species of the Carboniferous spiriferid genus

Reticularia (George, 1932, p. 535), of which *Reticularia mesoloba* is almost recti-marginate, *R. obtusa* is moderately uniplicate, and *R. lobata* is parasulcate. The development is illustrated in Fig. 7. Unfortunately, the time sequence of these forms is not certainly established, but it will scarcely be denied that such progressive morphogenesis indicates the true direction of evolution, even if collateral morphic equivalents are unwittingly introduced into the seriation. The essence of the illustration is of course contained in the fact that in its ontogeny *R. lobata* passes through a very brief rectimarginate stage and a uniplicate neanic stage before reaching the parasulcate ephebic form. That is, the neanic *R. lobata* is more similar to the adult *R. obtusa* than it is to the young *R. obtusa*. In still more advanced members of the same series, the advent of plication in ontogeny may occur sufficiently early to confine the rectimarginate stage to the extreme umbonal region, or may possibly eliminate it altogether (though conditions of preservation of the early shell make it difficult to be certain about this). Again it would appear that each species in its ontogeny recapitulates its phylogeny, and that pressing back of the earlier phyletic stages occurs in the ontogeny of the later forms, which may lead to lipopalingenesis.
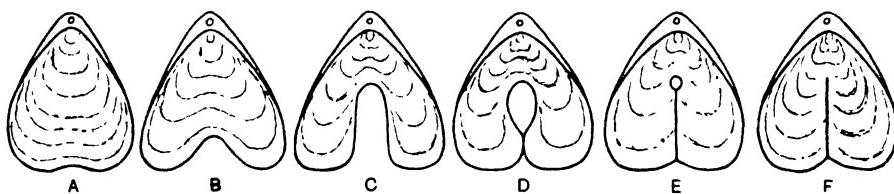
Fig. 8. Diagrammatic representation of the ontogeny and morphogeny of shell form in the diphyoid terebratuloids. Modified after Buckman.

A comparable instance is that afforded by the diphyoids, described by Buckman (1906). These terebratuloids fall into at least three parallel series, which Buckman has referred to different genera; the changes that occur in the shell shape are essentially the same in each, however. In the most primitive glossothyrid stage, the shell sculpture is alternate, with a sulcus in the dorsal valve complemented by a blunt carina in the ventral. In dorsal view, the anterior margin is slightly concave. Progression occurs in the accentuation of the concavity, so that the shell becomes divided into two lobes by a deep indentation. The lobes approach each other anteriorly and ultimately unite and fuse, the bilobate stage thus leading to a perforate stage. Further progress results in the decrease in size of the perforation by increased fusion, until finally the perforate stage of this amazing series disappears completely, and the forms become imperforate again, the only relics of the morphogenesis being the indentation of the anterior margin, and a cicatrix and incision along the middle line of each valve. Buckman suggests that even the latter may be lost, and the end form of this cycle of evolution be almost identical in external appearance with the initial progenitor. The changes are illustrated in Fig. 8.

The ontogeny of the later forms is of course clearly displayed by the growth lines, an examination of which immediately demonstrates that form *D*, for example,

passes through a bilobate stage after a glossothyrid stage before attaining the perforate stage. It is also clear that the perforate stage is attained in form E earlier than in form *D*. That is, the ontogeny of the later forms of these series recapitulates their morphogeny, and the morphogenetic stages are condensed tachygenetically. Also, while it is true that the median hole in forms *D* and *E* is scarcely a positive *biocharacter* (or unit of structure), yet its elimination from the ontogeny of form *F* is manifestly the expression of the same process that occurs in the Zaphrentids and the Liparoceratids, which has been called lipopalingenesis.

The case bears some analogy to that of *Fissurella*, cited above. For the acute bilobate stage may be compared with the marginally slit *Emarginula*, while the perforate stage is the analogue of *Fissurella*. Of the latter group, Garstang (1929, p. 96) remarks: "...there is no getting over the fact that the conversion of a slit into a hole sooner or later involves an act of discontinuity—a mutation [= saltation]." The same remark absolutely applies to the diphyoids—amongst which the immediate ancestor of the earliest perforate form must have been imperforate—when its unreality becomes apparent. For in no sense can the gradual approximation in phylogeny, any more than in ontogeny, of the anterior portions of the lobes be considered saltative (unless the saltations be so minute as to be unobservable); and to introduce a distinction between a stage when the lobes are an infinitesimal distance apart, and a stage when they just touch, calling the progress a saltation, is to elevate an insignificant change from a number of similar insignificant changes of a process that is essentially continuous to the rank of a profound and fundamental alteration in the mode of evolution, which most definitely it is not. In the same way, for example, the development of marginal indentations and perforations in certain members of the echinoderm family Scutellidae is a gradual process, both in ontogeny and phylogeny, saltation playing no apparent part; while a similar remark probably applies to the byssal hole of such a form as *Anomia*, or to the pedicle opening of *Discina* (a brachiopod genus that bears remarkably close analogies to *Fissurella* in this respect, as does its form ancestor *Trematis* to *Emarginula*).

IX. RECAPITULATION AND DEVIATION.

There is no gainsaying that the Laws of Development of von Baer contain much truth, and as in de Beer's (1930, p. 7) view "the biogenetic law abandons von Baer's principle of progressive deviation, or rather relegates it to the state of the caenogenetic exceptions," he is therefore inclined to assert that the Biogenetic Law is false. Indeed, his thesis is "an attempt to replace on the rails laid by von Baer the train of biological thought shunted off them by Haeckel." There are, however, some grounds for suspecting that the violation of the one law by the other is more apparent than real, and is largely due to the confusion of phylogeny with morphological classification (cf. Bather, 1893, p. 280). In the first place, von Baer established his laws at a time when a Meckelian conception of Parallelism was current, and as the latter is a theory that compares ontogeny with a morphologically graded series of contemporaneous adults, it is scarcely to be expected that the two series should

conform one with the other. It is very obvious that the grades of structure of living forms at the most but suggest ancestral phyletic stages, for, being contemporaneous, they are *ipso facto* evolved along divergent lines, despite the treatment of such grades as phyletic by certain people[1]. While it is no doubt theoretically possible for evolution to stagnate for a time, and the form of a descendant to be but negligibly changed from that of even a remote ancestor, normally this is not the case, and change with descent is almost universal. It is therefore, generally speaking, impossible to discover two living animals of which one merely oversteps the other in ontogeny. At some point or another the similarities between them will break down, and deviation will more or less profoundly contrast the adults. It is the expression of this fact that is embraced in von Baer's laws, though he did not employ evolutionary terms.

But von Baer went further, and in his second law remarked on the increasing specialisation of structures and structural relations as ontogeny becomes more advanced towards adult stages. This may be reinterpreted, replacing the morphological by the evolutionary concept, by the observation that two different animals possess similar ontogenies for a longer or shorter time according to their degree of genetic relationship. But this is to be expected if unmodified palingenesis occurs, for if two animals diverge in phylogeny from a common ancestry, and their ontogenies recapitulate the respective phylogenies, then the early stages of the ontogenies will be similar. An instance is provided by the reticulate spirifers. It will be recalled that morphogenesis occurs in *Reticularia* by the development of a parasulcate dorsal fold after uniplicate and rectimarginate stages. It would appear that along another line of development (George, 1932, p. 535), the uniplicate fold, before attaining marked prominence, becomes grooved along the middle line to form the sulciplicate *Reticularia elliptica*. The two divergent series may be represented as follows:

Reticularia obtusa   *Reticularia elliptica*

\ /

Reticularia mesoloba
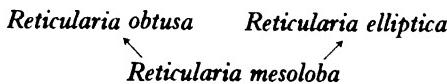
though it is only contended that *Reticularia mesoloba* represents a stage in the ancestry of the more advanced forms, and is not necessarily itself one of the ancestors. The ontogenies of both *R. elliptica* and *R. obtusa* pass through rectimarginate and feebly uniplicate grades, and to the latter they are similar, but afterwards they diverge as in phylogeny. A similar example is provided by the *Zaphrentis delanouei* group, in which *Z. lawstonensis* diverges from the *disjuncta* series (Carruthers, 1910, p. 534).

It is contended by de Beer and others that there may be (and generally is) a deviation between the ontogenies of the descendant and the direct ancestor. This is supposed to result from two main causes—paedomorphosis and acceleration. As to the latter, there is abundant evidence of its occurrence amongst fossils as a result of overstepping and tachygenesis. In a series with several biocharacters, the rates of

[1] See, for example, Crow (1926, pp. 92 ff.), in which it is expressly stated that a time sequence of fossil forms, such as elephants and horses provide, is an exactly similar phylogenetic series to that of the now-living morphologically graded Protozoa *Chlamydomonas* → *Gonium* → *Eudorina* → *Volvox*.

acceleration of the progressive biocharacters may not be uniform either in phylogeny or in ontogeny, so that the ontogeny of a late member of the phylogenetic series may contain total stages not represented by any adult ancestor, the stage of development of one biocharacter being more advanced, of another more primitive, than in a particular ancestor. Such relative acceleration, or precedentive palingenesis, is well known: an instance is supplied by the oxycones. It is probable that accurate measurement would show it to be of general occurrence. It is the only type of acceleration that will result in deviation, and the latter is not the kind of deviation emphasised by von Baer, who was more concerned with qualitative differences. The suggestion that this tachygenetic variation is due to different rates of operation of the genes, which again are presumed to be influenced by environment (and therefore can modify ontogeny irrespective of phylogeny), is little more than an unfounded speculation: it receives negligible support from an examination of the fossils, for the relative acceleration occurring in one evolving series may be reversed in another more or less parallel series in which the same biocharacters occur[1]. The principle of heterogony, too, is scarcely of general application: it may "explain" the horns of the titanotheres; it certainly does not explain the disappearance of the mandibular tusks in the descendants of *Tetrabelodon*, or the catagenesis of ornament in the ontogeny of countless groups. A more serious case of relative acceleration is that in which the later phylogenetic stages are so much telescoped that they appear in the earliest ontogenetic stages of the latest forms. Ontogeny then is not merely a reshuffled phylogeny: it is a beheaded phylogeny. Lipopalingenesis may result in the almost total separation of ancestor from descendant on morphological grounds, and in their ontogenies they are never even at a common point from which they may deviate; such are the cases of *Zaphrentis parallela* and advanced *Z. disjuncta*. When there is simple overstepping (*hypermorphosis*), ontogeny is a complete recapitulation of phylogeny.

Paedomorphosis in fossils is here intended to connote more than a relative acceleration of the development of the reproductive organs (neoteny), as occurs, par excellence, in the perennibranch salamanders; indeed, amongst fossils it is well-nigh impossible to apply this criterion, for sexual maturity is rarely accompanied by any permanent concomitant skeletal modification in any group. It includes two very distinct phenomena: bradygenesis, and true caenogenesis. The former is the counterpart of tachygenesis, and occurs in ontogeny as a retardation of a biocharacter or a stage to progressively later stages as phylogeny proceeds. Bradygenesis occurs rarely, so far as experience goes, but that it does occur is unquestionable, as is instanced by the Liparoceratidae. The numerous examples of bradygenesis (including neoteny and a narrowly defined deviation) which de Beer (1930, pp. 42 ff.) gives might be more acceptable if the account of the ontogenies was supplemented by some reasonably established, rather than surmised, phylogenies; in most cases, the evidence is derived from a comparison of contemporaneous types, and merits the criticism of von Baer's laws. One point must be emphasised: the bradygenesis that

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[1] For an analysis of this problem, see Swinnerton (1923, pp. 385 ff.); also Swinnerton (1921, p. 357; 1932, p. 322).

is known to occur in phylogeny—that is, in fossils—is not the prolongation into the adult life of a character or stage which is caenogenetic, which was, without question, inserted *de novo* into ontogeny during embryonic or nepionic stages and which bears no relationship whatever to previous phylogeny. Thus the continuance throughout Mesozoic ammonites of the stout depressed nepionic whorls may be an adaptive feature, but certainly their origin, so far as all the evidence indicates, was mutational and not saltative; and when, as in the Liparoceratids, that stage is retarded, the conclusion to be drawn is no more than that the relative rates of development of biocharacters in phylogeny may undergo change in ontogeny; it is not evidence of clandestine evolution, of the kind postulated by de Beer (1930, p. 30).

It would be absurd to deny the occurrence of true caenogenetic interpolations in the life cycle: there are countless instances of embryonic and larval characters which obviously never existed in any adult ancestor—which, indeed, if they did occur in any adult would render the creature non-viable: the amnion and allantois will serve as excellent examples of such features. Amongst fossils, instances are not so common. The spinous larval stages of certain trilobites, and the protaspis itself, have been considered as caenogenetic adaptations to a planktonic habit Swinnerton, in Raw, 1925, p. 322). On the other hand, other observers (Raw, 1927, pp. 9 ff.) contend that, even if modified, these stages are essentially palin-genetic, for it would appear that they certainly recapitulate tachygenetically such features as the eye-line and associated structures (Walcott, 1913, p. 706), and they are also of use in the recognition of family relationships, though the families are morphologically established on adult characters (Raw, 1927, p. 22). The disagree-ment about these trilobites is interesting, for it clearly illustrates one point: while in many cases (especially of living forms) it is evident that certain structures occurring in nepionic stages are not recapitulatory, yet not infrequently it is difficult to declare with certainty, even after exhaustive examination, that a particular character is truly caenogenetic. That is, in the last resort phylogeny is the only certain criterion of caenogenesis. The astonishing result of studying fossils (which, of course, are the only relics of phylogenies) is that in not one single instance, so far as I am aware, has it been established beyond question that a caenogenetic character, saltatively in-serted into the early stages of ontogeny, has been progressively retarded in the descendants of the caenogenetically affected ancestor until present in the adult. In other words, caenogenesis is not known to affect subsequent phylogeny, despite Garstang's opinion to the contrary.

There is a serious practical difficulty in recognising caenogenetic characters in fossils, for the implications of a general occurrence of the phenomenon are profound: they demand a determination of phylogeny that shall not be dependent upon either ontogeny or comparative morphology. Naturally, an ontogenetic criterion is im-possible, as the very introduction of a caenogenetic feature thereby causes the onto-geny to differ from the phylogeny. And comparative morphology is eliminated, not only because at some point in evolution the descendant will notably differ from the immediate ancestor by the saltative introduction of the caenogenetic character, but also because adult comparison, when the young stages are different, notoriously

leads to the pitfalls of homoeomorphous confusion, especially amongst such relatively simple structures as fossils. And the determination of phylogeny on other grounds than these is a matter not unattended with difficulty.

It also must be emphasised that a general occurrence of caenogenesis, especially amongst such (supposedly[1]) similar forms as *Culex* and *Corethra*, or the species of *Peripatus*, upon which Hurst (1893, p. 196) and de Beer (1930, pp. 42 ff.) place so much weight, is in direct opposition to the laws of von Baer.

X. CONCLUSION.

This article professes to offer little that is new. It is intended chiefly as a demonstration of the kind of evidence that palaeontologists, who are the " defenders of the Biogenetic stronghold," have used in deducing the following conclusions: In many instances, especially amongst living animals in which the soft tissues are preserved, there is an obvious departure in ontogeny from any conceivable phylogeny, and an interpretation of phylogeny based on ontogeny alone would lead to wholly erroneous conclusions. But in a large number of cases, ontogeny appears to be a straightforward recapitulation of phylogeny; when the latter cannot be precisely determined—when lineages are not unequivocal, but the supposed ancestry includes collateral morphological equivalents—ontogeny may at least recapitulate morphogeny. With such recapitulation, the essential ancestral stages are reproduced with little change (except in size) and in proper time sequence; evolution then appears to proceed by simple hypermorphosis or additive development. If recapitulation occurs, tachygenesis (p. 115) is inevitable, unless ontogeny is to be a process prolonged indefinitely. When tachygenesis results in congestion, it is sooner or later obliged to lead to lipopalingenesis (p. 115), whereby characters are either passed over at the beginning of ontogeny, or are eliminated from the middle of ontogeny when they represent a (palingenetic, p. 110) deviation from the direct developmental course from nepionic (p. 111) stage to ephebic (p. 111) stage. These modifications of hypermorphosis may occur alone, or be accompanied by heterochrony (p. 110), in which case the time-sequence of the arrival of palingenetic characters in phylogeny is obscured by relative acceleration or relative retardation. Finally, palaeontologists in practice rarely discover unquestionable evidence of the interpolation of caenogenetic (p. 110) characters which profoundly modify ontogeny, and it is suggested that in any case such characters are difficult to recognise by ordinary methods of phyletic analysis. These various divergences from a complete recapitulation may result in ontogeny bearing little resemblance to phylogeny—may, indeed, result in no phylogenetic stage being recognisable in ontogeny. But recapitulation is of too general occurrence to be explained merely by relegation to the incidental; and although no satisfactory causal relationship between ontogeny and phylogeny has so far been determined, yet it would appear that when ontogeny departs from phylogeny, a profound recapitulation has been modified, rather than that when ontogeny mirrors phylogeny, a superficial recapitulation has been imposed.

[1] But for a Haeckelian explanation, see Bather (1893, p. 281).

Very largely, these conclusions are not based on the *a priori* determination of phylogeny by recourse to ontogeny: the sequence of adults is established on other grounds (generally morphogenetic), and it is secondarily observed that the ontogeny of the later members of the series often recapitulates their phylogeny.

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THE PRESENT STATUS OF PLANT VIRUS RESEARCH[1]

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(With One Plate.)

CONTENTS.

I. INTRODUCTORY.

IN preparing this statement of the present position of the study of plant viruses the writer has been actuated by the following considerations. In the first place, as the time is not yet ripe for the production of a text-book on plant viruses, there is no

source of information on the subject for the student and virus worker other than the various papers scattered throughout the literature; it is therefore hoped that the present and the previous review (Smith, 1931 *a*) will fulfil this need. In the second place, a general survey of the subject may help to relate some of the many apparently contradictory facts to each other and thus reveal any new principles at present obscured by a redundancy of data. Thirdly, some attempt to clear up the present confusion concerning the potato mosaic group will be welcome to most plant virus workers and to plant pathologists generally. Finally it is hoped that a general survey of the position in the plant virus world may be of interest to the animal virus worker, who, to quote Dale (1931), "in unravelling what is still such a tangle of contradictions needs all the help that can be given by concurrent study of the analogous phenomena in plants."

It would seem that research upon plant viruses is in urgent need of a lead in some definite direction of enquiry; not much further progress is likely to arise from the continued study of symptomatology, to which too much attention has been given in the past. Undoubtedly the whole subject suffers for lack of a stable and intelligible system of virus differentiation, and the application of current methods of study to such differentiation is discussed in this article. It may be, however, that differentiation must wait upon the discovery of more facts concerning the true nature of viruses before it can be placed upon a stable and scientific basis.

II. CHIEF METHODS OF APPROACH TO THE PLANT VIRUS PROBLEM.

In the following section the most important lines of study are discussed and the outstanding facts achieved by their application are outlined.

A. PHYSICAL PROPERTIES.

Studies on the physical properties of plant viruses must of necessity be mainly confined to those viruses which are sap-transmissible; at the same time with developments in the technique of feeding insects upon solutions (see section C (3)) it may become possible to extend these studies in part at any rate to viruses which are only insect borne.

(1) *Reaction to heat, chemicals, etc.*

Heat. More attention has been paid to the virus of tobacco mosaic than to any other plant virus, and the effect of heat will be considered first upon this virus. It should be realised, however, that in these and other studies there is no guarantee that the various workers have been dealing with the same "tobacco mosaic"; indeed it is highly probable that several different viruses are involved. This is one of the serious disadvantages arising from a lack of means of differentiation and classification of plant viruses and this lack will be reflected throughout the present review. Allard (1916), who did much pioneer work on tobacco mosaic, found that the virus was quickly killed at temperatures between 80 and 90° C. It resisted higher temperatures when in a dry condition than when in solution, and did not lose infective

power at −180° C. with liquid air. Mulvania (1926 *a*) obtained 10 per cent. infection at 89° C., but no infection at 90° C. and over. James Johnson (1927) has studied the effect of heat on a number of tobacco mosaic viruses, nine in all. He found the thermal death-point to range from 60 to 90° C. for 10 min., the lowest temperature of 60° C. inactivated his mild tobacco mosaic (tobacco virus 3). Johnson (1926 *c*) found that the virus was attenuated by exposure to temperatures of 35–37° C. for 10 days. Henderson Smith (1928 *a*), working with tomato aucuba mosaic (probably Johnson's tobacco virus 6), states that this virus withstands heating for 10 min. at 80° C., but is inactivated at 90° C. Bewley (1927), working with tomato "stripe" which is of the tobacco mosaic type and may be a virus complex, states that heating for 10 min. at 80–90° C. destroyed its ability to produce mosaic disease by inoculation. Brewer *et al.* (1930) find that "typical" tomato mosaic is inactivated by short exposures to 88° C. and by longer exposures to 82–84° C. McKinney (1927 *b*) considers that the temperature at which the virus of tobacco mosaic becomes inactivated depends on the concentration of the virus and on the nature of the plant extract (see p. 146). He gives the thermal death-point to be 82–84° C. at dilution of 100 times and 88–90° C. when undiluted.

Turning now to the consideration of the effect of heat upon some other plant viruses, the thermal death-points appear as a whole to be lower than those of tobacco mosaic viruses. James Johnson (1929) gives the inactivation points to be 43° C. for crinkle mosaic, 70–75° C. for leaf-rolling mosaic, 40–45° C. for mild mosaic, 60–65° C. for rugose mosaic and 70° C. for the "mottle form" of rugose mosaic. Henderson Smith (1928 *b*), working with mosaic from various potato varieties, gives the thermal death-points at 70–80° C. It must, however, be borne in mind that many of the above potato diseases are due to virus complexes and not single entities (Smith, 1931 *c*; Murphy and M'Kay, 1932). Goss (1931), working with potato spindle-tuber and unmottled curly dwarf, obtained 30 per cent. infection after exposure to 75° C. for 10 min., but no infection at 85° C. The following are the thermal death-points of a number of other plant viruses: mosaic of bean, *Phaseolus vulgaris* (Fajardo, 1930 *b*), 44–56° C., but higher temperatures, 65–66° C., when in the seed; mosaic of dock, *Rumex obtusifolius* and *R. lanceolatus*, 80° C. for 10 min. (Grainger and Cockerham, 1930); tomato streak, above 80° C. (Doolittle and Blood, 1930); tomato spotted wilt, 42° C. for 10 min. (Bald and Samuel, 1931); yellow dwarf of onions, 75–80° C. for 10 min. (Henderson, 1932); tobacco ringspot, 60–70° C. (Henderson and Wingard, 1931); cucumber mosaic, 43° C. (Fajardo, 1930 *b*). As regards curly top of sugar beet which is not sap-transmissible, Carsner and Stahl (1924) consider that the virus is not destroyed by temperatures lower than those at which the beet tissue is killed. A glance at the foregoing list reveals the wide range in thermal death-points exhibited by the various viruses tested. Tobacco mosaic is the most resistant to high temperatures, while mild and crinkle mosaic of potato, spotted wilt of tomato and mosaic of beans are the least resistant. ✓

Chemicals. Allard (1918) tested the effects of a large number of chemicals upon the virus of tobacco mosaic. He found that the following had little effect on the virus: nitric, hydrochloric, phosphoric, citric, acetic and carbolic acids, manganese

sulphate, sodium chloride, aluminium sulphate, lithium nitrate, sodium nitrate, silver nitrate and mercuric chloride. On the other hand the virus was quickly destroyed by 80 per cent. ethyl alcohol, and 40 per cent. formalin. In an earlier paper Allard (1916) states that ether, chloroform, carbon tetrachloride, toluene and acetone fail to destroy the infective principle. Fukushi (1930) found that the virus of tobacco mosaic was remarkably resistant to various kinds of alkaloid, salts of alkaloid, glucoside and ethereal oil when it was subjected to those chemicals in the mosaic tobacco juice. Oil of mustard in 2 per cent. and digitalin in 5 per cent. destroyed the virus in 5 days. Nicotine in 1–2 per cent. and atropine in 1 and 2 per cent. concentration were toxic to the virus, while saponin, 2 per cent., had no appreciable effect. Klebahn (1931) treated tobacco mosaic with alcohol and glycerine and considered that it resisted these substances for several days. James Johnson (1926 c) finds that the virus of tobacco mosaic is quite sensitive to the inactivating influence of oxygen when properly exposed to it under moist conditions. A statement of Duggar (1929) may be quoted here: "The tobacco mosaic agency is relatively very resistant to changes in temperature and to such chemical agents as acids, alcohols and the salts of the heavy metals; in respect to inactivation it is therefore in a class parallel with enzymes, certain spores and some of the more resistant vegetative cells." Henderson Smith (1928 a) has investigated the effect of alcohol upon aucuba mosaic of the tomato (probably tobacco virus 6), and he has found that the virus is not inactivated by alcohol up to 90 per cent. As regards the effect of chemicals upon the potato virus group little work has been done on this subject. James Johnson (1929) considers that the potato mosaic viruses are comparatively sensitive to such chemical agents as alcohol, nitric acid and formaldehyde; 25 per cent. alcohol and 1 in 500 nitric acid inactivate the viruses of crinkle mosaic and rugose mosaic in 1 hour. On tobacco the potato rugose mosaic virus is destroyed by 1 in 200 formaldehyde in 1 hour, but the "mottle form" remains active; formaldehyde 1 in 100, however, destroys the "mottle form." Henderson Smith (1928 b), experimenting with mosaic from different potato varieties, found the virus to be less resistant to alcohol than aucuba mosaic of tomato, being inactivated by 90 per cent. after 1 hour's exposure and sometimes by 80 per cent. The virus from Majestic and Arran Chief showed higher resistance than that from Up-to-Date. The virus in filtered juice withstood "20 per cent. chloroform" for 4 hours at 27° C., and the dyes Auramine O and Meldola blue diluted 1 in 2000 for 2 hours at 27° C.; Acriflavin, 1 in 1000, did not wholly destroy it; Formalin 1 in 500 for 2 hours at 27° C. apparently killed it, but 1 in 1500 did not reduce its infectivity. Since the above work of Johnson and Henderson Smith it has been discovered that many potato mosaic diseases consist of complexes of viruses (Smith, 1931 c), and furthermore the writer has found (1932 b) that the virus known as *Y*, a common constituent of potato mosaic diseases, is not filterable under ordinary conditions through candles. It is necessary to remember these facts in considering the above experiments upon the effect of chemicals on potato mosaic viruses. Fajardo states (1930 b) that the virus of bean mosaic is sensitive to alcohol, no infection being obtained after treatment for 30 min. with 25 per cent. alcohol. Dock mosaic (Grainger and

Cockerham, 1930) is inactivated by 2 per cent. formalin in 30 min. but not by 95 per cent. alcohol in the same time. Some plant viruses will not stand drying, such as a tobacco ringspot (Henderson and Wingard, 1931) which is readily destroyed by desiccation as is also the potato mosaic virus known as Y; on the other hand certain tobacco mosaic viruses retain their infectivity for a period of years in dry tissues.

The effect of certain plant juices upon the infectivity of tobacco mosaic has been tested (Duggar and Armstrong, 1925). It was found that the sap of pokeweed, *Phytolacca decandra*, inactivated the virus at a dilution of 1 in 5; the juice of *Datura stramonium* was effective at a relatively high dilution (1 in 100), while that of geranium (*Pelargonium*) caused a reduction in the number of diseased plants from 20 to 8 and 3 at dilutions of 1 in 10 and 1 in 100 respectively. The juice of the following plants exerted no injurious influence: cotton, squash, potato, sweet potato and apple.

Investigations on the effect of enzymes upon the virus of tobacco mosaic have been made by Lojkin and Vinson (1931). Under the conditions of the experiments incubating the virus preparations with emulsin, pepsin or yeast extract did not reduce the infectivity. Trypsin, pancreatin and papain inactivated the virus; erepsin inactivated the virus only after an incubation period of several days. None of the enzyme preparations had any effect on the virus in fresh untreated juice, and the inactivating effect of the enzymes was destroyed by boiling. Some experiments on the effect of high pressure upon the tobacco mosaic virus (Giddings *et al.* 1929) have proved that while 75,000 lb. pressure had little effect, no infection developed in any plants inoculated with mosaic juice subjected to pressures of 130,000 lb. or over. The pressure required to inactivate tobacco mosaic virus is higher than the pressure death-point of a number of bacteria but lower than that of *Bacillus subtilis*. The enzyme zymase is inactivated at a lower pressure than that required to destroy the tobacco mosaic virus, the pressure death-point of which probably corresponds approximately to that which inactivates pepsin. In a recent note to *Science*, Olitsky and Forsbeck (1931) state that they have inactivated the virus of a mosaic upon tomato by pulverisation of the tissues.

(2) *Dilution.*

Allard (1914–15) found that the virus of tobacco mosaic was unaffected by dilutions up to 1 in 10^4 when attenuation was indicated. McKinney (1927 *b*), on the other hand, obtained 20 per cent. infection with dilution of 1 in 10^5. Using the aucuba mosaic of tomato, Henderson Smith (1928 *a*) obtained a 37·5 per cent. infection with 1 in 10^4, but no infection in greater dilutions. With regard to potato mosaics, these appear to be much less tolerant of dilution than viruses of the tobacco mosaic type; James Johnson (1929) considers that a dilution greater than 1 in 10 usually results in rapid diminution of the percentage of infection. Crinkle mosaic and mild mosaic are apparently the most readily inactivated by dilution, withstanding with difficulty dilution between 1 in 10 and 1 in 100. Leaf-rolling mosaic may still be quite infectious at 1 in 200; rugose mosaic, since shown to be a virus complex (Koch, 1931), apparently will not stand a dilution much greater than 1 in

100. The mottle form of the virus from apparently healthy potatoes will stand a dilution of 1 in 10³. Henderson Smith (1928 *b*), using mosaic from various potato varieties, found infection up to dilutions of 1 in 10³. In considering the results of these two workers in the light of new work it should be realised that the "rugose mosaic" of Johnson is a mixture of viruses, and so in all probability were the mosaic diseases used by Henderson Smith. Furthermore, while Johnson apparently used unfiltered juice and was thus dealing with more than one virus at a time, Henderson Smith used filtered extracts and by so doing eliminated the virus *Y* which was almost certainly present but which does not pass a Pasteur-Chamberland candle (Smith, 1932 *b*). It is probable that Johnson's mottle was the same virus as Henderson Smith's mosaic from Majestic and is of the *X* type (Plate I). Henderson Smith (1928 *b*) suggests that there may be a series of strains or variations of the virus of potato mosaic, and this question is discussed in section IV. The following are the dilution end-points of some other viruses: potato spindle tuber, 50 per cent. infection at 1 in 10³; unmottled curly dwarf, 10 per cent. infection at 1 in 10³ (Goss, 1931); tobacco ringspot, infectious at dilutions of 1 in 10³ but only a trace of infection at 1 in 10⁴ (Henderson and Wingard, 1931); bean mosaic gives no infection at dilutions higher than 1 in 10³ (Fajardo, 1930 *b*); dock mosaic gave positive infections at 1 in 10² but not at 1 in 10³ (Grainger and Cockerham, 1930).

The reason for the inactivation of plant viruses by dilution is one of considerable interest; the loss of infective power may be explainable merely on statistical grounds by dispersal of particles or it may be a definite inactivation due to dissociation of the virus particle from its colloidal support. The first point to be settled then is whether dilution is a reversible process, and there do not seem to be any references in the literature to experiments of this kind; the writer is at the moment investigating this matter.

(3) *Purification, filtration and adsorption.*

It is manifestly impossible in a review article to deal adequately with such complicated subjects as those of filtration and adsorption. It must suffice therefore to make a general survey of the filterable properties of plant viruses and to try to indicate their relationship to the questions of purification and adsorption; attention will be directed to certain points of special importance. For a good general account of filtration the reader is referred to chapter II in Rivers' *Filterable Viruses*, contributed by Stuart Mudd. Before adequate studies can be made of the filterability, longevity *in vitro* and other properties of plant viruses the plant juices contained in the virus must first be cleared of extraneous matter and the virus concentrated as much as possible. There are various methods of thus clearing and concentrating virus solutions. Firstly, by the use of high-speed centrifuging (McKinney, 1927 *a*); Barnard and Elford (1931) have shown that certain animal viruses are of very high density and are easily concentrated by means of centrifuging. Secondly, by means of precipitation by chemicals; Vinson and Petrie (1931) have devised a method for the precipitation from solution of the virus of tobacco mosaic by means of a solution of lead acetate, since the safranin precipitate method was found not to be favourable

for the isolation of a product free from pigment. By the use of this method Vinson and Petrie claim that they can obtain a virus solution equal in infective power to the original juice but containing only about 1 per cent. of the total solids in the original juice sample. It is not known how far this method is applicable to other viruses. In a recent abstract, Vinson (1932) states that the safranin precipitate of the tobacco mosaic virus has been satisfactorily decomposed by the addition of Lloyd's alkaloidal reagent. After the removal of the safranin the supernatant liquid is usually more highly infectious than the original juice. Brewer *et al.* (1930) prepared clear and colourless suspensions of "typical" tomato mosaic virus practically free from the various constituents of the plant juice by discarding the liquid from the super-centrifuge and suspending the gummy residue in distilled water, centrifuging again and clearing and decolorising the resultant liquid with aluminium-gel acid in reaction. McKinney (1931 *b*), working with various green mosaics in tobacco, removed extraneous solids and soluble materials from the virus extracts by centri-fuging and temperature coagulation. Henderson Smith (1928 *a*), using filtered juice of tomato with aucuba mosaic, found that all the virus was brought down by a precipitate when 60 per cent. alcohol was added. The supernatant liquid was found to be inactive after filtration. Similarly Duggar (1929) found that $CaCl_2$ when added to tobacco mosaic juice produced a precipitate which carried down the virus.

Vinson and Gildenhaus (1932) state that the tobacco mosaic virus can be pre-cipitated from the juice of diseased plants by the addition of a solution of lead acetate. After washing the precipitate with an $M/3$ solution of primary potassium orthophosphate, the virus can be freed from the precipitate by means of a dilute solution of potassium hydrogen phosphate, the reaction of which is nearly neutral. Such a solution, according to Vinson and Gildenhaus, is very infectious and contains Kjeldahl nitrogen, which is absent from a solution of healthy plant juice.

Reference must here be made to the work of Elford (1931), which will be more fully dealt with in a later paragraph. By means of two collodion membranes, the pores of one of which are just sufficiently large to allow passage of the virus while the pores of the other hold it back, Elford has been able to concentrate the virus on the second membrane after eliminating all bacteria upon the first; further washing of the virus residue on the second membrane removes much of the protein material present. This is an excellent method of concentrating and purifying animal virus solutions, and there seems no reason why the method should not be applied to the purification of plant viruses.

Turning now to the question of filtration of plant viruses through various filter candles, it will be seen how wide is the range of filterability of the different viruses by this method. Tobacco mosaic and other viruses of this type seem to be the most easily filterable by candle methods. Iwanowsky (1892) first showed that this virus would pass through a filter candle; since his time the tobacco virus has been filtered on very many occasions and is capable of passing various grades of the Pasteur-Chamberland candles and Berkfeld filters. The following is a brief survey of the filterability of a number of plant viruses as measured by the candle method: Henderson Smith (1928 *a*) found that aucuba mosaic of tomato was easily filterable

through an L_3 Pasteur-Chamberland candle. The writer, using another tomato mosaic, has obtained positive infections with the virus after passage of all grades of Pasteur-Chamberland candles from L_1 to L_{13} (1932 *a*). Potato viruses of the X type are filterable through L_1 and L_3 and occasionally L_5 candles (Smith, 1932 *b*). Folsom (1925–6) reports inactivation of potato rugose mosaic by passage of the coarsest grade of Berkfeld filter. The writer has recently shown (1932 *a*) that the Y virus, which is probably one of the constituents of the American rugose mosaic complex, will not pass an L_3 Pasteur-Chamberland candle. This point is further dealt with in the section on adsorption and collodion membranes. Henderson and Wingard (1931) state that their tobacco ringspot virus is filterable through a Berkfeld filter of W grade, provided the juice is first freed of its suspended solid matter, while Priode (1928), dealing with apparently the same ringspot virus, finds that it will not pass a Berkfeld filter grade N. The two cases that follow deal with viruses that are not sap-transmissible. By the use, however, of the insect-feeding technique (p. 152), Storey (1932 *a*) has demonstrated that streak of maize at pH about 6 passes Chamberland candle L_1, Berkfeld V and N filters, less freely through Chamberland L_3 and not at all through a Seitz EK filter disc. By similar means Severin and Swezy (1928) have shown that the virus of sugar beet curly top is filterable through coarse, medium and fine Berkfeld candles. The viruses of the following diseases are apparently not filterable by candle methods. The filters used in each case are indicated in brackets. Mosaic of dock (Jenkins filter, English Berkfeld candle, Grainger and Cockerham, 1930); mosaic of bean (coarse Berkfeld filter, Fajardo, 1930 *b*); calico disease of potato (Porter, 1931); bunchy top of tomato (L_1 Pasteur-Chamberland candle, Seitz EK 6, McClean, 1931); spotted wilt of tomato (Pasteur-Chamberland L_1, L_3, L_5, Smith, 1932 *a*); Y potato mosaic virus (Pasteur-Chamberland L_3, L_5, Smith, 1932 *b*). It is unlikely that the failure of these viruses to pass the filter candles is due to the size of the respective particles but probably to adsorption, and here it will be opportune to consider the question of adsorption in relation to filtration. In many ways the filter candle is unsuitable for use in plant virus studies, the chief drawback being the great bulk of material which is liable to adsorb the virus, and this, in the writer's opinion, is the reason why many viruses are not filterable by these candle methods. That plant viruses may differ widely in their capacity for adsorption is shown by the writer's studies of the potato mosaic viruses known as X and Y (1932 *b*). If plant sap containing these two viruses be passed through an ordinary Kieselguhr bed in a Büchner funnel, the filtrate will contain a pure "culture" of the virus X, the Y virus having adsorbed to the Kieselguhr. Again, by filtering a similar X and Y complex through an L_3 Pasteur-Chamberland candle, the X virus is found alone in the filtrate. It is in this connection that the work of Elford (1931) and of Barnard and Elford (1931) is likely to be helpful; this work is indeed a definite advance in the study of filterable viruses, and although so far it has been applied only to the study of animal viruses there seems no reason why it should not be equally applicable to plant viruses, though certain modifications of method may be necessary owing to the different nature of plant and animal juices. Briefly stated, Elford has developed a method for preparing collodion membranes

called "Gradocol" membranes, of uniform pore size, the size of the pore being graded at will by varying the constituents and technique of preparation. By measuring the rate of flow of water the average pore diameter can be calculated and from these the size of the virus particles is deduced. In calculating the size of the virus particle from the size of a membrane pore it must be assumed firstly that the membrane pores are parallel-sided and secondly that the virus particles are not plastic. The writer has succeeded in filtering the potato virus Y through such membranes, although it would not pass the L_3 Pasteur-Chamberland candle (see also MacClement and Henderson Smith, 1932). There have been attempts some-what on these lines by other workers to measure the size of virus particles, notably by Mulvania (1925 b), by Duggar (1921) and Klebahn (1931), but these workers have mostly used percentage collodion filters which are not made with the same accuracy as those of Elford. In a recent statement Waugh and Vinson (1932) consider the particle size of the virus of tobacco mosaic to be less than $5\mu\mu$ in radius.

Although there may be more than one opinion as to the accuracy of virus particle size as measured by Elford's membrane, yet this is not of fundamental importance and his methods seem to open up new avenues of approach to the virus problem. Firstly the membranes afford a means of filtering viruses which are not filterable by means of candles, secondly by using a membrane which will just hold back the virus, the latter may be purified and concentrated; and thirdly it may be possible to show that virus diseases hitherto thought to be single entities are due to mixtures of units. In a recent letter to *Nature* (1932), MacClement and Henderson Smith state that such a separation of two viruses has already been brought about; in addition they give the approximate particle size of various plant viruses as measured by means of Elford's membranes. In the writer's opinion Elford's membranes, the use of enzymes, and selective transmission by insect vectors will reveal the composite nature of more than one virus disease.

(4) *Longevity* in vitro.

It is perhaps in their reaction to simple ageing *in vitro* that plant viruses show the widest variation. At one end of the scale is the virus of tobacco mosaic which remains infective for periods of years, while at the other end are the viruses of cucumber mosaic and tomato spotted wilt which remain viable only for a period of hours in extracted plant juices. The following records show the longevity *in vitro* for several plant viruses. In tobacco mosaic the active principle was found to be still viable in leaves that had been dried and preserved for 24 years (Connecticut, 1927). Dickson (1922) states that on May 25th, 1925, two leaves of each of four healthy tobacco plants were inoculated from mosaic tobacco plants, kept since February 1920; on June 15th each of the four plants showed symptoms of tobacco mosaic. Johnson (1926 c) finds that tobacco mosaic virus may retain its vitality for several months in soil and considers that its duration of life is much shorter in sandy than in clay soils, and this may be determined by the oxygen relations. Holmes (1928 a) stored the virus of tobacco mosaic under different conditions and found that virus kept at freezing-point lost less strength than the samples kept in a bottle

at room temperature. He concludes that a virus does not retain all of its strength over long periods of time. As regards other viruses of the tobacco mosaic type, Henderson Smith (1928 *a*) found that filtered juice of aucuba mosaic tomato plants was still active after being stored in subdued light at room temperature for a year or more. According to Doolittle and Blood (1930) a form of tomato streak remains viable for at least 180 days, and the writer working with a tomato mosaic (1932 *a*) finds it still viable after several months. Pierce and Hungerford (1929) offer evidence that the bean mosaic virus is capable of remaining viable in the seed for a period of 30 years. On the other hand some viruses are very short-lived outside the plant host. Johnson (1926 *a*) considers that cucumber mosaic retains its infectiousness for less than 2 days in liquid tobacco extract. Henderson, W. J. (1932), states that the virus of "yellow dwarf" of onions is inactivated after 112 hours in sterile water at 29° C., while that of tomato spotted wilt becomes inactivated after 4–6 hours ageing (Bald and Samuel, 1931; Smith, 1932 *a*). As regards potato viruses, Goss (1931) finds that the viruses of spindle tuber and unmottled dwarf are inactivated rapidly after being extracted from the plant. The writer has found with regard to the *X* and *Y* potato mosaics that the longevity *in vitro* is 6 weeks for *X*, so far as tested, and 24 hours for *Y*.

It is not known whether the inactivation of a virus *in vitro* may not be partly caused by changes taking place within the plant juices which contain the virus. Thus one possibility is that such inactivation is caused by oxidation, certain viruses may be very much more susceptible to oxidation than others, and this may be one reason for the extremely short life *in vitro* of the viruses of spotted wilt, cucumber mosaic and others.

(5) *Effect of light on plant viruses.*

In this section it is the effect of light upon the *virus* itself in extracted juice which is considered and not the effect of light on the *disease* which is dealt with in section II B (1). There are three papers dealing with the effect of ultra-violet light upon the virus of tobacco mosaic which are of importance. Mulvania (1926 *a*) exposed the extracted juice of mosaic tobacco plants to ultra-violet light for periods of $\frac{1}{8}$–4 hours; his results indicate that exposure of 1 hour or more inactivated the virus. Fanny F. Smith (1926) in similar experiments found that the shorter or abiotic ultra-violet rays inactivated tobacco mosaic in filtered juice after 30 min. exposure, while under similar conditions a suspension of *Bacillus prodigiosus* was killed in 30 sec. This is considered to be against the theory that the causal agent of tobacco mosaic is a living organism. On the other hand some more recent work seems to disagree with these findings. Arthur (1928), in experiments on radiation from a mercury vapour arc in quartz, found, when using appropriate filters, that the injury to the plant tissue begins at a wave-length shorter than 285 mμ and increases rapidly with decreasing wave-length, the killing time of direct exposure of virus without filters being less than 15 sec. Arthur and Newell (1929) state that the virus causing tobacco mosaic was found to be completely inactivated in 15 sec. exposure to the open arc when the virus was prepared sufficiently free of contaminating

material. These workers also state "the evidence shows only that a virus whatever its nature can be inactivated when sufficiently free from contaminating material by an extremely short exposure to ultra-violet radiation." Further experiments showed that the virus could be killed with a short exposure (1 min.) when spread upon the leaf surface. If irradiated the day following inoculation there was no appreciable killing of the virus. It is apparently impossible to inactivate the virus when it has penetrated far into the plant tissue, although irradiations were given of sufficient intensity and quality to kill the whole upper surface of the leaves. Dickson (1922) carried out a number of experiments on the effect of light on the virus of tobacco mosaic; he tested the effect of red, green and blue light, sunlight and darkness upon the extracted sap of diseased tobacco plants. He considered that there was attenuation of the virus in the vials kept under blue, red and green light in descending order as given, but there is little difference of moment between the red and the blue. Darkness and green light were approximately similar in their effect.

Before concluding this survey of the methods of studying the physical properties of viruses it is necessary to mention a paper by Walker (1926) ("A comparative study of the mosaic diseases of cucumber, tomato and *Physalis*"). Put briefly, Walker's thesis is that the physical properties of a plant virus vary according to the species of plant host; were this to be the case it would be obvious that many of the above methods of study would be rendered nugatory. Walker says (*loc. cit.* p. 454): "In short, the so-called properties of the mosaic virus seem to be determined by the character of the juices of the plant host in which the disease occurs." McKinney (1925) also appears to favour Walker's views to a certain extent, for he says: "Plant juices are complex in nature, and it is not known just how much influence they may exert on the behaviour of a given virus when subjected to different treatments." With these two exceptions the general opinion of plant virus workers is that the physical properties of a virus are inherent and do not depend in any appreciable degree upon the species of plant host. It is probable that the apparent variations in the properties of a virus according to the host, as described by Walker, can be explained on the basis that he was working with mixtures of viruses (Johnson, 1927).

B. SYMPTOMATOLOGY.

(1) *Appearance of diseased plants.*

The symptoms produced by virus diseases in plants are so varied that only the more outstanding types can be referred to here. Although symptoms are unreliable and misleading, the virus worker cannot afford to dispense with them as a diagnostic character. The appearances of virus-diseased plants may be classed broadly into groups. The first and largest group are known as the "mosaics"; here the leaves of affected plants exhibit mottling of various shades of green or yellow with or without leaf distortion and sometimes numbers of concentric rings on leaf or stem. Secondly, there is the "yellows" group, where the whole leaf is more or less uniformly coloured; *e.g.* aster yellows. Thirdly, there is a type of symptom in which the leaf is distorted, as in potato leaf roll, tomato fernleaf, etc. Fourthly, the whole

plant may be affected, as in the "rosette" type of symptom, "bunchy top" of bananas, "curly dwarf" of potato. Fifthly, there is a clinical picture in which necrotic spots, streaks or stripes may occur on the leaves and stems of affected plants. And lastly, there is a miscellaneous group of symptoms such as Klebahn's "alloiophylly" of anemone, cranberry false blossom, spike of sandal, spindle tuber, spindling sprout, giant hill of potato, little peach and many others.

The weakest point in the use of symptoms as a diagnostic character is the fact that the same virus can cause different symptoms according to the kind of host plant. A good case in point is the disease known as tomato spotted wilt. This virus has three main types of symptom according to the species of plant it is affecting and the time the plant has been affected. Thus on *Solanum capsicastrum* the symptoms take the form of numerous concentric rings (Smith, 1931 b), on tobacco it may appear as a lethal form of streak, while on a third host it is a mosaic only. In considering symptoms therefore as a diagnostic character it is essential to use as long a series of differential hosts as possible in order to study the symptom range of a particular virus. The use of differential hosts in virus diagnosis has been developed by Johnson (1926 a), the writer (1931 c) and others, and this method is further dealt with in section III.

It will be necessary to consider briefly the effect of the environment upon the symptom picture of plant virus diseases. As regards the effect of temperature it is no uncommon thing to find the symptoms suppressed or masked at high temperatures. Johnson (1922), working with potato mosaic, finds that temperatures as low as 6° C. do not inhibit the disease, for which the optimum temperature lies between 14 and 18° C.; above 20° C. all symptoms disappear. Similarly with tobacco mosaic (Johnson, 1921) the optimum for the activity of the virus lies between 28 and 30° C., while if severely affected plants are grown at temperatures of 36–37° C. for about a fortnight the newly developing leaves are free of mosaic mottling. Goss (1924), working with mosaic potato, variety Bliss Triumph, states that "mottling was found to be the most constant symptom when temperature alone was varied. It disappeared under the combined influence of high temperature and intense sunlight." In a later paper (Goss and Peltier, 1925) it is stated that "air temperature was again found to be the most important factor studied in inhibition or masking of the foliage symptoms." Other references to masking of potato mosaic symptoms by high temperatures are to be found in the work of Tompkins (1926), Dobrozrakova (1927), Schultz and Folsom (1923) and others. The foregoing cases deal with the suppression of symptoms by high temperatures, but Clayton (1930), working with mosaic of crucifers, finds that at 70–80° F. the mustard and Chinese cabbage developed a severe streak while other plants were much stunted. At 55–65° F. even the more susceptible plants made fair development, while mosaic brussel sprouts and cauliflowers often ceased to show symptoms and grew normally. Carter (1927 b) also states with regard to sugar beet curly top that high light intensity and high temperature appear to favour the development of severe symptoms. As regards the effect of light upon the symptom picture Shapovalov and Lesley (1931) have studied in some detail the effect of shading on the rate of development of tomato "yellows"

(= curly top of sugar beet): their data indicate that shading increases the tolerance of the plants to the virus. If continued after the occurrence of infection shading enables the plants to produce a crop and even facilitates complete recovery in some cases (see section II H). This fits in with the observations of Carter above, who found that high light intensity favoured the development of severe symptoms. In another paper Shapovalov (1931 *a*) finds that 4–6 hours' exposure to four 500 watt Mazda lamps accelerated the rate of disease development which was retarded by shading. Sreenivasaya (1930 *b*) considers that the masking of spike disease of sandal is influenced by a combination of intense sunshine and high temperature. It is known that environment may affect the development of symptoms in the case of virus diseases of the potato, especially mosaic and leaf-roll, there being a difference between the symptoms evoked by the same virus in the same potato variety in a glass-house and out-of-doors respectively, and it has been observed that some varieties which "carry" a virus without symptoms under glass may be visibly diseased when grown in the open. Here it is opportune to say a word of warning as to the use of certain fumigants when studying potato virus diseases under glass. The writer has repeatedly found that the use of tetrachlorethane, or fumigants containing this substance, produces upon potato plants symptoms which cannot be differentiated from the virus disease known as leaf-roll, and it is probable that in the past the effect of tetrachlorethane has been mistaken for genuine leaf-roll and insects have thus been incriminated as vectors which do not in truth disseminate this virus.

(2) *Pathological histology.*

Microscopical examination of virus-affected plants reveals two main kinds of pathological conditions: there are firstly the more obvious necroses and tissue alterations wrought by the virus, and secondly the characteristic and controversial cell inclusions which have been called X-bodies. The last-named will be dealt with first; in a recent paper in this *Journal* Henderson Smith (1930 *b*) has reviewed the whole subject of these X-bodies and reference will therefore only be made here to one or two important papers on the subject which have appeared since Henderson Smith's survey. The first of these is by Sheffield (1931) and deals with the formation of cell inclusions in various Solanaceous hosts affected with aucuba mosaic of tomato. Sheffield describes in detail the formation of the X-bodies, which consist apparently of aggregations of minute protein particles. These particles fuse and form large masses which are carried passively about the cell; there is no evidence of any autonomous movement. Sheffield considers that these inclusions are not organismal in nature, they seem to be products of reaction of the host cell to the virus, but they may contain the etiological agent of the disease. Later Clinch (1931) published an account of cytological studies of potato plants affected with certain virus diseases. Clinch found abnormal inclusion bodies in the chlorotic areas of potato leaves in association with simple mosaic, interveinal mosaic, crinkle and streak. They were not seen in aucuba mosaic or leaf-roll; the X-bodies were most abundant in interveinal mosaic and crinkle plants suffering from prolonged effects of the disease, but they were inconspicuous in young diseased plants. Clinch suggests that the

X-bodies arise as a result of viscosity changes in the cytoplasm of the diseased cells and should be regarded in consequence as an effect and not a cause of the disease. Fukushi (1931) describes intracellular bodies associated with the dwarf disease of rice and considers them similar in nature to the X-bodies described from other plant virus diseases. It cannot yet be said whether the X-bodies associated with plant viruses are analogous with the cell inclusions which are associated with certain animal viruses such as infectious ectromelia of mice (Barnard and Elford, 1931).

As regards the other pathological effects there seem to be certain histological changes in the tissues which are common to those mosaic diseases which do not produce necrosis. Firstly, there is a difference in lamina thickness between the dark green and chlorotic areas of the leaves, the dark green being as a rule thicker (Dickson, 1922; Clinch, 1931; Cook, 1931 a). This appears to be due to the inhibited development of the cells in the pale areas (Clinch, 1931). Exceptions are found in corn mosaic and sugar-cane mosaic, however, where the light green or chlorotic areas are thicker than the dark green areas (Matz, 1919; Kunkel, 1921). Secondly, the chloroplasts tend to be reduced in number and size, while in acute infection they may break up into small hyaline granules (Dickson, 1922). This latter point may have some bearing upon v. Brehmer's supposed causal organism (section II D); there is also a tendency to starch accumulation. With regard to the abnormal dark green areas in mosaic-affected leaves, Dickson (1922) finds that the cells are larger than normal and contain more chloroplasts and the chlorophyll itself is darker in colour. Dickson's conclusion is that in the mosaic diseases studied by him the tissues directly affected are chlorenchymatous and the vascular tissues are apparently unaffected, but this statement presumably would not hold good for those mosaic viruses which produce a mosaic mottling on some hosts and necrosis on others, such as happens with many potato mosaic viruses. Thus Bawden (1932) states that a potato mosaic virus from the variety Di Vernon causes a necrosis of the phloem in certain varieties but a mosaic only on others, and in these latter the tissues are found to be normal. Quanjer (1931 a) has described and classified a number of necroses due to potato viruses; he gives five types of necrosis: (1) *Phloem necrosis*, this is restricted to the phloem and is characteristic of leaf-roll. (2) *Top necrosis*, radiating from a small percentage only of the internal phloem and seldom from the external phloem. (3) *Acropetal necrosis*, chiefly in the collenchyma of the leaf veins, petioles and stems. (4) *Pseudo-net necrosis*. (5) *Concentric necrosis*. The last two types of necrosis are confined to the potato tuber and occur in the storage parenchyma. Gilbert (1929) also describes a necrosis of the potato tuber known as *net necrosis*, which consists of the appearance of a network of brown strands in the vascular region of the tuber, involving the sieve tubes, companion cells and adjacent cells of the phloem parenchyma. This is considered to be a first-season symptom of leaf-roll and has only been described in America. In a recent paper Bawden (1932) describes three types of necrosis of potato-plant tissue due to virus infection. (1) *Acronecrosis* (= top necrosis); internal symptoms are produced in the petioles, stems and tubers and consist of necrotic changes which originate in the phloem and spread thence to all other tissues. (2) *Acropetal necrosis* (leaf-drop streak) occurs on

the stem and petioles affecting chiefly the collenchyma, the vascular tissue being normal. (3) *Phloem necrosis* (leaf-roll); necroses confined to the phloem elements and consist of lignification. Quanjer (1913. *Meded. Landb. Hoogesch.*, Wageningen, 6, 41) considers that the rolling of the leaves in this disease is brought about by the necrosis of the phloem, but Murphy (1923) and Bawden (1932) consider that the necrosis is secondary to the rolling, as the latter may be very pronounced in the first season when the former is absent or very faint. Rochlin (1930) finds that destructive necrosis of the phloem is associated with rugose mosaic, curly dwarf and stipple streak as well as with leaf-roll, and considers that the diseases in question produce generalised and not merely local effect.

C. METHODS OF VIRUS TRANSMISSION.

There are, broadly speaking, five methods by which plant viruses can be transferred or transmitted from diseased to healthy plants; two of these may be regarded as artificial, while the other three occur in nature.

(1) *By grafting.*

Of the artificial methods the first to be considered is transmission by *grafting*. So far as the writer is aware, all plant viruses are so transmissible, and there are certain viruses or infectious chloroses which appear to be transmitted only by this means; these will be dealt with later. There are various methods of grafting; in the case of potatoes and similar plants the method largely used at the Cambridge Plant Virus Station consists of inserting the scion, the stalk of which has been cut into a wedge shape with a sharp scalpel, into a slit in the stem of the stock; the two are then bound lightly with fine rubber tape, the end of which is cemented with ordinary rubber solution. There are other methods of grafting in use in virus studies which have been developed or adapted by workers to suit particular circumstances. Sreenivasaya (1930 c) has described two methods which he finds suitable for the transmission of "spike" disease of sandal; these are "patch-grafting" and "leaf-insertion." In the former a piece of bark including the cortex and the bast and either with or without buds is detached from the diseased scion and transferred to healthy stocks; in the latter method, which, strictly speaking, is not grafting, but is included here for convenience, a fresh diseased leaf is trimmed to a rectangle, a "window" is opened in the bark of the healthy stock, the leaf is inserted, the flap or window is closed and the whole bandaged with wax cloth. Harris (1932) has described methods of grafting strawberry plants by "cleft-grafts" and two kinds of "inarching." An adaptation of the tissue insertion method of grafting has been developed by Murphy and M'Kay (1926) for infecting potato tubers with viruses. This consists in boring a hole in the healthy tuber with a cork borer and inserting a plug from the diseased tuber cut with a cork borer a size larger; the inserted plug of tissue may or may not have an "eye" in it. A similar method of plugging has been used by Bonazzi (1926) for transmitting sugar-cane mosaic. As already mentioned, there are certain virus diseases which so far as present knowledge goes are only transmissible by grafting; these are the infectious chloroses of *Abutilon*, etc., spike

of sandal, peach yellows, mosaic of *Sida rhombifolia* (Kunkel, 1930) and possibly the potato mosaic virus known as "paracrinkle" (Salaman and Le Pelley, 1930).

(2) *By inoculation.*

The second method of artificial virus transmission is that of *inoculation*, using this term in its restricted sense of actual application of the virus to the plant tissue. Inoculations can be performed by means of hypodermic injection, by needle scratches or by rubbing the leaves with muslin or filter paper soaked in virus extract. Holmes (1929), Samuel (1931) and others have shown that many plant viruses are more efficiently transmitted when the leaf to be inoculated is lightly rubbed with the virus so as to cause slight superficial injury rather than laceration. There are modifications of these inoculation methods, and Sein (1930) has developed a method of artificial inoculation of sugar-cane mosaic which he claims gives 94 per cent. infection. This consists of stripping the central spindle of the outermost leaf, binding the cylinder of tender leaves thus exposed with a mosaic leaf and pricking through this with a number of insect pins mounted in a handle. In a few cases the more infectious plant viruses can be disseminated by means of a cutting or pruning knife in the ordinary course of tending the plants (Wilbrink, 1930). B. Johnson and Duggar (1930) claim to have produced 80 per cent. transmission with "typical" tobacco mosaic by stomatal infection. Their method was to spray the healthy plants with virus extract by means of an atomiser. It is difficult to reconcile these results of Johnson and Duggar with the statements of Caldwell, firstly that the exudate from the hydathode does not contain virus (1931), and secondly (1932) that he has injected the intercellular spaces of the leaf of *Nicotiana glutinosa* with virus juice and no infection took place unless cells were accidentally ruptured. A possible explanation of Johnson and Duggar's results is that the leaves may have been bruised or excoriated by insects, thereby allowing entrance to the virus.

(3) *By the agency of insects.*

Turning now to natural means of spread, the first and most important method is by the agency of *insects*. As the whole question of the relationship of plant viruses to insects has recently been reviewed by the writer (1931 *a*), only one or two papers which have appeared since that review was written will be mentioned here. Storey (1932 *b*) describes a new virus disease of tobacco which is transmitted by a species of Aleyrodes, probably *Bemisia gossypiperda*; this disease is characterised by enations of the veins and curling of the leaves. Thung (1932) describes a virus disease of tobacco in Java known as "kroepoek," which is similar to that described by Storey, and is also transmitted by a species of Aleyrodes which appears, however, to be a species other than *Bemisia gossypiperda*. Kirkpatrick (1931) has published an important paper on further work with leaf curl of cotton and its transmission by *Bemisia gossypiperda*. Bald and Samuel (1931) and the writer (1932 *a*) have made further studies on spotted wilt of tomato and the insect vectors which are two species of Thrips, *Frankliniella insularis* and *Thrips tabaci*. Linford (1932) has described a virus disease of pineapples known as "yellow spot" which is transmitted by *Thrips tabaci*.

In both these diseases there is found to be a delay in the development of infective power within the Thrips and this is probably due to a necessary multiplication of the virus within the body of the insect. Bald and Samuel (1931) and Linford (1932) have made the interesting discovery that it is necessary for the Thrips to feed in its larval form upon an infected plant in order to become a vector of the virus; the adult insect cannot pick up the virus *de novo*. Moore (1932) describes a disease of tobacco in Africa transmitted by *Thrips tabaci*; in the writer's opinion it is possible that both the disease described by Moore and "yellow spot" of pineapple may be due to the same virus as that causing spotted wilt of the tomato. Drake *et al.* (1932) state that "yellow dwarf" of onions is transmitted by *Aphis rumicis* L., *Aphis maidis* Fitch, *Rhopalosiphum prunifoliae* Fitch, and the six-spotted leaf-hopper *Cicadula sexnotata* Fall. Blattný (1931) considers that it is possible to detect a difference between the salivary glands of aphides (*Myzus persicae*) which have fed upon leaf-roll, mosaic and stipple-streak potato plants and of those of uninfective individuals. The difference is stated to lie in the areola around the cell nucleus of the salivary glands which is dark in infective insects instead of clear. Storey (1931), continuing his studies upon streak of maize and the leaf-hopper *Cicadula mbila*, describes the inheritance by the leaf-hopper of ability to transmit the virus. The character of ability to transmit was found to behave in inheritance as dominant to inability to transmit and a further series of studies of crosses between the two pure-breeding races showed that the character is sex-linked. Storey's results accord with the *Drosophila* type of sex-linked inheritance. Hayes (1932), working with rosette of groundnuts in the Gambia, thinks the insect vector may be *Aphis laburni* in addition to *Aphis leguminosae* as shown by Storey and Bottomley (1928).

It will be convenient to consider in this section a recent contribution to the technique of feeding insects on virus or other solutions (Carter, 1927; Storey, 1932 a; Hamilton, 1930). In these experiments Fife (1932) has substituted a thin section of paraffin wax for the membrane used by Carter. Pieces of glass tubing, 1·5 cm. diameter and 2 cm. in length, served as the cage for containing the insect, in this case a leaf-hopper. One end of the cage was covered with cheese cloth which was held securely in place by a rubber band. A section of paraffin ribbon, cut 60 microns thick with a microtome, was stuck to the other end of the cage after the leaf-hopper had been inserted; a drop of solution was then placed on the paraffin wax and the insect allowed to feed. A further development was designed by Fife consisting of two cells on a slide to enable the feeding to be observed under the microscope.

(4) *By seed.*

Transmission of virus diseases by the *seed* is not of common occurrence, but there are certain authentic cases where this does occur, and these will be shortly discussed. Perhaps the best known and most widely studied case of virus transmission by seed is that of mosaic of bean, *Phaseolus vulgaris*; as high as 50 per cent. infection may sometimes develop in commercial seed of susceptible varieties (Fajardo, 1928). At the same time the infection of the seeds is irregular, some seeds only in the same pod being affected. Ray Nelson (1932) considers that an explana-

tion of these peculiarities of seed transmission and localisation of symptoms may possibly be found in the assumption that the virus is restricted to certain tissues, and that localisation of infection prevents or retards dissemination of the causal agent to all portions of the plant. Further, supposing that the virus of bean mosaic moves through the vascular tissues, the failure of some seeds to become infected with the virus can be explained by the type of arrangement that characterises the vascular tissues of the pod. In the writer's opinion the current theory that all plant viruses are systemic within the plant host is likely to be modified in the near future. There is already a fair amount of evidence for supposing that in certain cases the virus may become localised in the plant, resulting in a few foci of infection only. Some such theory is necessary to account for certain phenomena in the potato mosaic group, and the writer has observed this on several occasions with the virus of spotted wilt in some host plants, while Henderson Smith records a similar occurrence (1930*b*).

For further information on the question of transmission of bean mosaic by seed the reader is referred to the work of McClintock (1917), Reddick and Stewart (1918), Kendrick and Gardner (1924), Burkholder and Müller (1926), Merkel (1929), and Pierce and Hungerford (1929). Although the question of seed transmission of potato viruses is one of considerable economic importance, little work seems to have been carried out on the subject. Elze (1931 *c*) considers that he has evidence that leaf-roll and aucuba mosaic of the potato are carried in the seed. The study of this problem in the potato plant, however, is complicated by the distortions, mottlings and streak-like symptoms which develop in potato seedlings, due apparently to chromosome abnormalities. Duggar, in a paper on the problem of seed transmission of the "typical" mosaic of tobacco (1930), draws attention to the probability of the relation of transmission to adsorption and inactivation through storage proteins. An experiment of the writer's in regard to *Solanum nigrum* infected with the potato mosaic virus *Y* may be relevant to this. Seeds were extracted from the ripe berries of such an infected *S. nigrum* and divided into two portions; one portion was washed for periods of 3–6 hours in running water while the remainder was stored for some weeks in an envelope at room temperature. After the given period of washing under the tap, the first lot of seed was ground up in a mortar and the resulting paste inoculated into White Burley tobacco plants; these inoculations gave a high percentage of infection in the tobacco plants. After a period of some weeks the other portion of the same seed, now completely dried, was also ground up in a mortar and inoculated similarly to tobacco; these inoculations were negative in every case. The experiment seems tò indicate that the virus *Y* was either adsorbed to the outside coat of the seed and could not be removed by washing or was enclosed within the seed coat and will not stand desiccation, and so the fact of keeping the seed till it was dry completely inactivated the virus. It may be added that seed from infected berries of *S. nigrum* always produced healthy plants. It is also of interest in this connection to find that the juice of *ripe* tomato fruits apparently inactivates the virus of spotted wilt (Bald and Samuel, 1931; Smith, 1932 *a*).

The following is a list of some other plant viruses which are considered to be transmitted through the seed: mosaic of *Dolichos biflorus* (Uppal, 1931), mosaic of beet (Ducomet, 1929), tobacco ringspot by seed of petunia only (Henderson, 1931), a tobacco ringspot by seed of Turkish tobacco (Johnson, E. M., 1930), mosaic by seed of *Pisum sativum, Trifolium pratense, T. hybridum, Melilotus alba* and *Hippeastrum* sp. (Dickson, 1922), "dot" mosaic by seed of lettuce (Brandenburg, 1928; Newhall, 1923), two ringspot diseases of tobacco (Valleau, 1932). As regards tomato viruses, Bewley and Corbett (1930) are of opinion that tomato mosaics (and also cucumber mosaic) are seed-transmissible, while Berkeley (1931) offers evidence of the transmission of tomato streak by seed in Canada. Finally the reader is referred to a useful bibliography by Orton on "Seed-borne parasites" (1931).

(5) *By pollen.*

The last method of virus transmission to be considered is by the *pollen.* There is only one plant virus in which this mode of transmission is described, this is bean mosaic, and Reddick (1931) offers evidence in support of the transmission of the virus by this method. Transmission by pollen has been suggested on more than one occasion in order to explain the spread of certain virus diseases of which no insect vector can be discovered, notably spike of sandal, peach yellows and others. Transmission by the pollen would explain some points in the spread of certain potato diseases of the X-type which are almost universal in commercial crops of potatoes but which do not appear to be transmitted by the aphides and other common insect fauna of the potato. There is, however, no evidence as yet of such a mode of transmission of these viruses.

D. Attempted cultivation *in vitro* of a plant virus and some supposed causal organisms.

In 1924 Olitsky considered that he had demonstrated the multiplication *in vitro*[1] of the virus of tomato mosaic in that he was able to obtain infection with the twelfth subculture in sterilised tomato juice. Since that time a number of virus workers have attempted to repeat this work of Olitsky, notably Mulvania (1925 *a*), Goldsworthy (1926), Purdy (1926), Henderson Smith (1928 *a*) and Grainger (1929); all these attempts to cultivate the viruses of both tomato and tobacco mosaics have failed. Goss (1931) has also carried out experiments with negative results on the attempted cultivation of the virus of potato spindle tuber. In 1931 Olitsky and Forsbeck made a fresh investigation of Olitsky's original work, and their conclusion is given here: "In nearly every case, irrespective of the method employed, there was some increase in the potency of the virus beyond the original titration, as was also observed by McKinney. In the early experiments this increase was much greater than in the later ones, possibly on account of some cyclic variation in the virus. The question arises whether the increase was due to an activation or a dispersion of aggregated virus particles by some unknown mechanism, or whether it represented actual multiplication of a living agent. *The latter interpretation cannot be definitely*

[1] *in vitro* = cell-free media.

accepted on the basis of the present data." It can therefore be stated that up to the present no plant virus has been cultivated *in vitro*.

Turning now to the consideration of some supposed virus organisms which have been described from time to time, these may be grouped under the headings of Protozoa, bacteria and fungi. Some work by Ray Nelson (1923) attributed the virus of bean mosaic to a protozoan, while Eckerson (1926) gives a description of a flagellate organism associated with tomato mosaic. Several authors have associated bacteria with virus diseases. Ray Nelson (1932) describes a small coccus in the seeds of mosaic beans and considers that bacteriological studies in connection with the results of seed transmission tests indicate a close association between the coccus and the virus. Ray Nelson was unable, however, to reproduce the disease of bean mosaic by inoculation with these bacteria. In a recent letter to *Nature*, Bewley (1931) considers that he has evidence connecting a tomato mosaic with a bacteriophage and puts forward the suggestion that the mosaic virus is a bacteriophage adapted to a free life within the tomato plant. Swezy and Severin (1930) also consider a bacterium to be associated with curly top of the sugar beet. Other writers who have described bacteria in connection with the etiology of plant viruses are Melhus (1922), Boncquet (1916) and Smith and Boncquet (1915).

Finally v. Brëhmer and Bärner (1930) and v. Brëhmer (1931) state that they have discovered the causal organism of potato mosaic, possibly of potato leaf-roll and of beet mosaic. They describe yellowish green, oval amorphous bodies which they consider are organisms belonging to a hitherto unknown systematic group of the Archimycetes allied to the Plasmodiophoraceae. In his paper v. Brëhmer (1931) states that the causative organism of potato mosaic and possibly potato leaf-roll is a Myxomycete, *Plasmodiophora solani*, while that of beet mosaic is *P. solani* var. *betae*. In most of the above cases it must of course be assumed that the "causative organism" is capable of existing in a filterable stage. In considering the relationship of these varied organisms and the plant viruses with which they are associated it is clear that much more research is needed before it can be said that any close connection between the two has been demonstrated.

E. MOVEMENT OF THE VIRUS WITHIN THE PLANT HOST.

The movement or translocation of viruses within the plant has recently been fully discussed by Henderson Smith (1930 *b*), and the writer therefore proposes to deal only with certain advances in the subject made since that review. Caldwell (1930) has traced the movement of the virus of aucuba mosaic in the tomato plant. In the test plant a middle portion of the stem was killed either by steam or chloroform, leaving a bridge of dead tissue between the upper and lower portions of the plant, and inoculations were made either below or above this bridge. In neither case did the disease develop in the portion of the plant on the other side of the bridge. The results, which indicated that the virus agent was not travelling in the xylem stream, led Caldwell to believe that the translocation of the aucuba mosaic virus in the tomato plant takes place in the living ground tissue and in the phloem. In a later paper, Caldwell (1931) has demonstrated that normally the virus agent does not

enter the water stream of the plants, but, if introduced experimentally, it is carried round in the xylem vessels, from which it cannot pass into the ground tissues and phloem unless the vessels are mechanically injured. All the indications are that the agent cannot enter an unbroken cell or move into or out of dead cells. By counting the local lesions formed by tobacco mosaic on the leaves of *Nicotiana glutinosa* and taking this as an indication of the virus concentration, Holmes (1930) has studied the local and systemic increase of the tobacco mosaic virus in the host. The mosaic virus developed to a high concentration near the site of inoculation in a leaf of *N. tabacum* before reaching measurable concentration in other portions of the inoculated leaf or in other parts of the plant. A slow spread of virus through the tissues of the inoculated leaf accompanied the increase in concentration near the site of inocula-tion, and appeared to be independent of the rapid spread which carried virus to distant parts of the plant. This local increase and slow spread of the virus constitute a local or primary phase of the disease. The systemic or secondary phase of the disease was marked by the nearly simultaneous appearance of increasing quantities of virus in the petiole of the inoculated leaf, in all portions of the stem, in the developing top leaves of the plant and in the roots with later invasion of old leaves. In a series of plants all successfully inoculated in similar leaves at the same time, the local increase of virus within the tissues of the inoculated leaf blade occurred simultaneously in all plants, but systemic spread of virus with its attendant mottling of developing leaves occurred early in some individual plants and late in others.

Holmes has also developed a method of iodine staining which renders local lesions conspicuous (1931) and reveals the points of infection. He found evidence that under the conditions of the experiments the virus in the lesions was locally present in high concentrations. Holmes's method not only constitutes a useful means for the investigation of the movement of the virus in its host but is also suitable for determining accurately the properties of a virus on a quantitative basis. Samuel (1931) has applied these methods in studying inoculation with a number of different viruses and he finds that temperature, age of the leaf inoculated and other factors influence the character and number of lesions formed. Price (1930) has shown that certain varieties of garden beans (*Phaseolus vulgaris*) react with local lesions to rubbing with a virus of tobacco mosaic. Price has prepared curves indi-cating the possibility of using the number of lesions on susceptible bean varieties as a measure of virus concentration. It is not clear whether Holmes's iodine methods are applicable only to viruses which give local lesions but, if starch accumulation is due to the presence of the virus in that spot independently of the lesions, then his method should apply to all viruses.

F. METABOLISM OF VIRUS-AFFECTED PLANTS.

The literature dealing with the chemical changes taking place within plants affected with virus diseases is now immense, and only some of the more important papers can be dealt with here. As regards potato leaf-roll, Barton-Wright (1932) finds that the disease slows down the formation of sugars in the leaf during photo-synthesis, while the rapid rise in the sucrose curve towards the end of the day is very characteristic. It appears as if the sucrose is unable to escape from the laminae

and Barton-Wright suggests that translocation in a leaf-roll plant takes place in the form of a slow leakage of hexose, instead of the normal sucrose, to the tubers via the ground parenchyma. Schweizer (1930), working on the same disease, considers that the diseased foliage is characterised by a copious accumulation of sugar in the form of glucose, and this abnormal sugar accumulation extends to the tuber. Schweizer states that microchemical tests of the stem of diseased plants revealed the absence of certain peroxidases, while Rouzinoff considered (1930) that oxidases and peroxidases were present in excess. Rouzinoff also found that the *p*H values of leaf-roll juice indicated that it was more acid than normal, but this does not agree with the work of Robertson and Smith (1931), who give a *p*H of 5·80 and 5·64 for healthy tubers of Ally and Arran Comrade as compared with 5·85 and 5·70 respectively for leaf-roll tubers. As regards the respiration of potato plants affected with leaf-roll, Whitehead (1931) states that respiration is higher in a diseased immature tuber, about the same in diseased and healthy tubers after prolonged storage, lower in diseased sprouting tubers but much higher than in the healthy plant once leaves have developed; this seems to differ from a conclusion of Dunlap (1930), who states that there is an increase of respiration in young tissues infected with virus, while in older diseased leaves respiration was *less* than in healthy leaves. Dunlap may, however, be referring only to mosaic viruses. Müller (1932), studying the assimilation of leaf-roll potato plants, found the width of stomatal apertures to be less in diseased than in healthy plants, but the respiratory intensity per unit area was approximately equal in both series. Carbon dioxide assimilation was found to be much reduced in diseased as compared with healthy leaves. In tomato plants affected with aucuba mosaic Bolas and Bewley suggest (1930) that the action of the virus on the starch is the production of acids which (1) react with nitrogen to form proteins, (2) attack the chlorophyll causing mottling, (3) affect respiration. There seems to be a difference between the chemical changes taking place in mosaic-affected plants and those in plants suffering from the "yellows" type of disease; thus mosaic diseases according to Dunlap (1930) are accompanied by an increase in the total nitrogen and a decrease in the total carbohydrate content of the foliage, while the reverse is true in the case of yellows. As regards sugar-cane, the starch-forming powers of mosaic cane appear to be reduced in proportion to the amount of infection while translocation is practically unimpaired (Cook, 1926). Cook differentiates mosaic diseases, *i.e.* those in which the photosynthetic activities are greatly reduced, from such diseases as peach yellows, little peach and potato leaf-roll by the fact that starch is formed in the leaves but translocation is affected with the result that the leaves become hard and leathery. Working with spinach "blight," True and Hawkins (1918) find that the starch content of diseased spinach tops is more than double the normal. In the roots the total sugar and starch were alike in diseased and normal plants. It appears that in spinach "blight" the process of carbohydrate manufacture is not inhibited though it may be retarded. Freiberg (1917) states that carbohydrates are more abundant in the dark green areas of mosaic leaves than in the light green, regardless of the time of day. According to Rosa (1927), leaves of tomatoes affected with "western yellow blight" (= curly top of sugar-beet) show a decrease of total nitrogen,

while reducing sugars, sucrose and starch increase progressively with the external symptoms. On the other hand, Shapovalov (1931 *a*), working with tomato yellows, which is apparently the same as "western yellow blight," finds an increase in the total nitrogen accompanied by a rapid accumulation of carbohydrates. Bewley and Bolas (1930) state that a marked reaction has been found to result from mixing the expressed juice of a tomato plant affected with aucuba mosaic with an aqueous colloidal solution of tomato chlorophyll. The most striking manifestation of this reaction is the development of a brown colour and the apparent destruction of a greater or less amount of the chlorophyll. The observations suggest that the reaction may provide a rapid and quantitative means of studying *in vitro* the nature of the virus and its reactions. Caldwell, however, considers that the virus of aucuba mosaic of tomato does not destroy the chlorophyll but tends rather to inhibit its formation.

A series of studies upon the changes wrought by the spike disease of sandal upon the host have been carried out by Sreenivasaya and his colleague (1929) and some of the salient points are noted here. The diastatic activity of saps from "spiked" leaves is definitely greater than that of healthy sap; there is a deficiency of calcium in the diseased as compared with healthy leaves and the *p*H differs from 5·15 and 5·71 in sap from healthy leaves to 4·69–4·99 in sap from "spiked" leaves; the total nitrogen in spiked leaves is generally greater than in healthy leaves. Sreenivasaya (1930 *a*) has recorded the presence of mannitol in the spiked leaves of sandal which he considers is one of the metabolic products of the virus.

G. Photography of viruses by ultra-violet light.

By means of his ultramicroscope and by the use of ultra-violet light, Barnard (Barnard and Elford, 1931) has been able to photograph the virus particles of several animal viruses. So far, however, Barnard has not applied his methods to the photography of plant viruses, though one may be permitted to hope that this may yet be accomplished. In the plant world the only attempt on these lines seems to be contained in a short paper by Holmes (1928 *b*), who used the apparatus devised by Köhler, which is constructed to allow the sorting out of light of a low wave-length from the cadmium spark and the isolation of a relatively pure beam of light corresponding to the wave-length of 275 millimicrons. This light is led through a series of quartz prisms and passed through the microscope, which is provided with quartz lenses adjusted for this exact wave-length. Holmes used a representative series of seven plant viruses, and juices from plants affected with these viruses were photographed with ultra-violet light of wave-length 275 millimicrons. No "formed structures" other than those seen in corresponding fluids from healthy plants were found.

H. Some other aspects of the behaviour of plants affected with virus diseases.

Under this heading four types of reaction are briefly touched upon. These are *recovery*, "*carrying*" *power*, *resistance* and *immunity*. As regards the first-named there are several apparently authentic cases of plants which have recovered from virus diseases, and these records are given herewith. Kirkpatrick (1931), in further

studies of leaf-curl of cotton, states that he has observed at least one case of complete recovery from the disease; Bennett (1930) reports complete recovery of Columbian raspberry plants affected with alpha-curl virus; Kunkel (1924) finds recovery of certain varieties of sugar-cane from mosaic a common occurrence; Barber (1928) has also recorded recovery from mosaic in the case of sugar-cane of the thick tropical or noble type. Murphy and M'Kay (1932) state that they have observed cases of recovery from crinkle and streak in potato (Kerr's Pink). Storey and McClean (1930), in studying transmission of streak between maize, sugar-cane and wild grasses, found that the maize virus frequently caused transitory infection of Uba cane in the form of a few large chlorotic streaks on the leaves. All evidence indicated that the cane plant made a complete recovery from this transitory infection and ceased to harbour the virus. The writer, experimenting with a virus from lupins, has found that tobacco plants are susceptible to the disease and show a characteristic mosaic; these symptoms rapidly fade and the plant regains its normal appearance. Transmissions from these plants have so far given negative results. The following are references, only, to further cases of recovery from virus disease: Verwoerd on two cases of recovery from a mosaic disease of tomato plants (1929) and Brierley (1916) on the same subject; Kunkel on grass mosaic in sugar-cane (in Rivers, 1928); James Johnson (1925) and Valleau and E. M. Johnson (1930) record instances of recovery of potatoes affected with true tobacco mosaic. In working with a similar virus on potato the writer has found that it does not become systemic in certain potato varieties (see also Blodgett, 1927). In all these cases the plants in question were apparently susceptible to reinfection with the same virus.

The phenomenon of "carrying" a virus disease without symptoms occurs commonly; the best example of this is to be found in the potato plant, some varieties of which habitually bear one or more concealed viruses. The same thing occurs with the viruses of the hop, and certain sugar-beet plants are capable of carrying the virus of curly top. Some plants may give an initial reaction on infection and later carry the virus without symptoms. It is probable that in the case of the potato the "carrying" power may break down under certain circumstances; it does not necessarily follow that a potato variety will always remain a carrier of a specific virus, nor does it follow that a potato plant will carry a specific virus under all environmental conditions. Virus carriers are of great economic importance as they act as sources of infection to other non-carrying plants or species, and this is especially well illustrated by the case of certain hop varieties. The subject of virus carriers in plants is one of great interest, but unfortunately practically nothing is known of the causes underlying the phenomenon, or whether the "carrying" power can be altered by changes in environment or by alterations in the virulence of the virus carried. As regards the latter point the writer has some evidence to show that the potato mosaic virus X, which is frequently carried by some potato varieties, can be increased in virulence by progressive passage through, or prolonged sojourn in, the tobacco plant. If this more virulent X be returned to a healthy potato plant of the "carrying" variety, it is found that the apparent "balance" has been disturbed and the more virulent virus is no longer carried without symptoms.

Salmon and Ware (1932) have found that inoculation of a chlorotic disease to hops which are carrying mosaic, induces definite mosaic symptoms and causes the breakdown of the carrying capacity.

Some degree of resistance to a virus occurs commonly among individuals of plant species which are normally susceptible to that virus, and it is to the production of a virus-resistant variety that one must look for the solution of the virus disease problem in economic crops. Resistance has been widely studied in the case of sugar-cane and its mosaic disease; thus the varieties known as P.O.J. 213 and 228 are largely resistant to the mosaic virus. Brandes (1931) describes some varieties of a new sugar-cane species, provisionally named *Saccharum robustum* Jesw., a marked characteristic of which appears to be freedom from mosaic coupled with vigorous growth. In crossing *Saccharum spontaneum* (immune) with *S. officinarum* (susceptible), Brandes finds that susceptibility appears to be recessive. On the other hand, Porter (1930), working with cucumber mosaic, finds in crossing the resistant variety Chinese Long with the susceptible White Spine that susceptibility to mosaic is dominant. In studies upon curly top of the sugar beet Carsner and Stahl (1924) find that in every severely affected beet field a few individual beet plants stand out conspicuously less affected than the rest. Mackie and Esau (1932), working on the susceptibility of beans to curly top, find that highly resistant varieties were found in the species of common beans and in the small Limas. Resistance to curly top is partially correlated with pink and red colours. Combination of resistance to both curly top and mosaic is possible in a single variety of bean. As regards potato virus diseases a recent statement in *Science* reports the production of a potato variety, known as Katahdin (1932), which is thought to be resistant to mild mosaic. Through the courtesy of Mr Stevenson, the writer has received a small supply of this potato for experimental purposes, and it may be of interest to record some preliminary inoculation experiments with this potato. The viruses used in the experiments are those known as X and Y (Smith, 1931 c), and they were inoculated to the plants by rubbing and by needle scratch. It was found that both viruses gave pronounced local symptoms on the inoculated leaf, round necrotic lesions in the case of X and streak-like lesions on the veins in the case of Y. Further development of the X-infections took the form of a mild mosaic mottling on the topmost leaves, while the other leaves developed a curious network of faint necrotic lines most pronounced on the under-surface of the leaves and unlike the reaction of other potato varieties to this virus. The behaviour of the Y virus on Katahdin was interesting in that the virus apparently did not spread beyond the inoculated leaf and so did not become systemic. No further symptoms developed and it was not possible to obtain the Y virus from other parts of the plant. It therefore appears that the Katahdin does not become systemically infected with the potato mosaic virus Y; it should be realised, however, that these experiments have been carried out during one season only. The resistance to virus shown by certain plants or plant varieties is often more apparent than real, and consists merely in the fact that for some reason which may be purely mechanical the plant is avoided by the insect vector and so escapes infection; this is almost certainly the case with some potato varieties. Similarly the

resistance to mosaic shown by some varieties of sugar-cane is thought to consist of a capacity on the part of those plants to recover rapidly from the disease and produce buds free of virus (Tims and Edgerton, 1931).

Reference to virus resistance in other plants may be found in the work of Lesley (1931), Fajardo (1930 *a*), Tims and Edgerton (1931), McClean (1930), Nelson (1932), McKinney (1923), Elmer (1927), Rankin (1927), Bennett (1928), and Lee (1928).

Plants do not apparently develop an immunity, and this fact is made clear in the section dealing with recovery where most of the plants which had recovered from the virus disease were again liable to infection. In this connection some observations on sugar-cane mosaic may be of interest. East (1931) records the fact that in sugar-cane there may be as many as three successive infections with, and recovery from, mosaic, recovery occurring at any time between 3 and 24 months after infection. East considers that there are two interpretations of this, either the infective agent has been killed or it may have been reduced in virulence until the symptoms were masked and the plant became a carrier. East is inclined to the opinion that an apparent immunity is acquired by a reduction of virulence of the mosaic virus. While it is probably true that there is no *acquired* immunity[1] to viruses in plants, certain species of plants do appear to be immune to a specific virus which affects other closely allied members of the same group. Thus the potato mosaic virus known as *Y* seems unable to infect the Solanaceous plant *Datura stramonium* (Smith, 1931 *c*), although this plant is very susceptible to another potato mosaic virus *X*.

Before concluding this section it will be convenient to deal here with three papers on immunologic reactions of tobacco mosaic virus. Purdy (1929) experimented with the reactions of the rabbit to inoculation with tobacco mosaic juice, and she obtained some fairly definite results. Separate antisera were produced in rabbits to normal sap from healthy Turkish tobacco plants and to virus sap from mosaic plants. The results of Purdy's experiments showed that the normal and virus saps of tobacco possess common antigenic substances and also that the virus saps of tomato, pepper and petunia possess antigenic substances in common with the virus sap of tobacco which are absent or present only in small quantities in normal tobacco sap. Purdy found that appropriate quantities of the antiserum to the virus sap of tobacco are capable of completely inactivating the virus sap. In a later paper Beale (1931) (Helen Purdy) states that no specific precipitate for virus extracts of tobacco affected by ringspot or cucumber mosaic was demonstrable. Beale considers that the specific antigenic substance in the virus extract of tobacco mosaic is foreign antigenic material, possibly virus itself, rather than altered host protein. Matsumoto (1929), working on the antigenic properties of tobacco mosaic juice, has studied the serological behaviour by means of precipitin reactions. The antigens used for the test consisted of (1) ultra-filtered mosaic juice and (2) centrifugalised supernatant liquid of the expressed mosaic juice without ultra-filtration. To each of these solutions was added an equal part of the antiserum at varied dilutions. Results of readings indicated that precipitation occurred in the antiserum-

[1] See Price, page 178.

virus mixtures of the two different preparations. No specific precipitation was observed when either the normal serum or the normal tobacco juice was used. Tobacco plants were inoculated with the supernatant liquid and the precipitate of the antiserum-mosaic juice mixture. It was found that the infective principle of the virus had disappeared in every case from the supernatant liquids of the anti-serum-virus mixtures, whence it may be inferred that the infective principle is precipitated by a specific action of the antiserum. Normal rabbit serum was found to be definitely incapable of destroying the infective quality of the virus, which was detected practically unimpaired in the supernatant liquid of the normal serum-virus mixtures as well as in the precipitate of the same kept in contact for 48 hours. In this connection a paper by Mulvania (1926 *a*) is of interest, this worker being unable to recover the virus of tobacco mosaic from the normal blood of a living rabbit.

I. ELECTRIC CHARGE OF VIRUS PARTICLES.

Takahashi and Rawlins (1930) have carried out experiments to determine the electrical charge of the tobacco mosaic virus, the results of which showed that the unpurified virus migrated to the anode in an electrical field at *p*H values between 4 and 9; no migration occurred at *p*H values between 3 and 1·2. Olitsky and Hoffmann (1930) have recently made a study of tomato mosaic with the objects of noting (1) the possible migration of a plant virus in an electric field, (2) the direction of migration, (3) any difference in behaviour of filtered and unfiltered suspensions. The results show that the mosaic virus, or particles containing it, migrate to the anode in an electrical field at *p*H readings of 5·3–8·5, the tomato virus therefore agreeing in this respect with most viruses of mammalian origin and with bacterio-phage. Bolas (1930) has also experimented on the movement of the virus of tomato mosaic in an electric field, and his tentative conclusions were that the portion of the virus producing severe symptoms were only to be found in the middle and towards the positive pole, while a portion capable of producing only mild symptoms was to be found on the negative side of the mid-point.

III. THE APPLICATION OF SOME OF THE FOREGOING METHODS OF STUDY TO THE DIFFERENTIATION OF PLANT VIRUSES.

Much of the confusion at present existing in the study of plant viruses is due to the fact that writers have attempted to name the disease instead of the virus, and also because they have not kept sufficiently clear in their minds the distinction between the disease and its causative agent. The time has now come to attempt to name the virus itself and not the disease it causes, which may differ according to the host on which it happens to be discovered. Owing to this wide range of symptom expression a single virus may be—and probably has been—described several times under separate names by different workers. This difficulty would be obviated if the virus itself were to be considered. This suggestion has been made already in a circular letter sent out by James Johnson and headed "Tentative suggestions for activities of the International Committee for co-operation in the study of description and nomenclature of plant virus diseases."

Just as it is not possible to identify a bacterium by means of the microscope alone, so it is unwise to attempt to identify a virus only by the symptoms it causes. There have been in the past various attempts to classify and differentiate plant viruses; thus James Johnson has differentiated a number of viruses (1927) by means of certain physical properties such as longevity *in vitro*, reaction to heat, alcohol, etc., and Quanjer (1931 *a*) has recently put forward a classification of potato viruses which is based upon the morbid anatomy of the potato plant. It seems to the writer that while both these methods are good, in themselves they are not sufficient; in other words, it is necessary in order to differentiate plant viruses to use both these methods and others as well. Johnson and Hoggan (1931) and Henderson Smith (1930 *a*) have stated the present position as regards the classification of plant viruses in papers read before the Fifth International Botanical Congress at Cambridge, and they also emphasise the importance of differentiating the virus entity rather than the disease it causes. The first step then towards this differentiation is the collection of as much information as possible about each virus, and not the disease only, by the methods which have been outlined in this review, *i.e.* (1) physical properties; (2) (*a*) host range and symptoms, particularly the symptom expression on differential hosts, (*b*) pathological histology; and (3) modes of transmission. In order to illustrate how these various methods can be applied to virus differentiation the writer has selected a few examples, choosing viruses which have somewhat similar symptom expressions. As the first examples two tomato viruses often occurring together will be considered: these are tomato spotted wilt and a tomato virus of the mosaic type. Applying some of the physical properties tests to these two viruses, it will be found that if plant sap containing both entities is allowed to stand in the laboratory for 24 hours, subsequent inoculation from it gives only the mosaic virus, as the longevity *in vitro* of spotted wilt is under 6 hours while that of the other virus is many months. Similarly, passage through a Pasteur-Chamberland candle shows that while the tomato mosaic virus is easily filterable, that of spotted wilt cannot pass a filter candle. There are also differences in the respective reactions to heat of the two viruses. Examining next the symptom expression of the same two viruses, it will be found that one particular type of symptom, *i.e.* the formation of numbers of concentric rings, the Liesegang phenomenon (Hedges, 1932), is associated with the spotted wilt virus and not with the mosaic virus. The method of examination by the use of differential hosts is also applicable. Thus on petunia species the spotted wilt gives local lesions and seldom becomes systemic, while the tomato mosaic gives no local lesions and produces systemic mosaic disease characterised by upward cupping of the young leaves. Finally, on the question of transmission, the two viruses are both sap-inoculable though they differ considerably in degree of infectiousness, but it is in respect to their insect vectors that they can be best differentiated. Thus in England spotted wilt is spread by *Thrips tabaci* but tomato mosaic is not so transmitted; indeed the actual insect vector of the latter virus does not appear to be known.

Considering now the above tomato mosaic and another closely similar tomato mosaic known in England as "stripe," it will be found that these two viruses

resemble each other closely in their physical properties and in their apparent non-transmission by insect vectors. On the other hand they can be distinguished by their reactions on the tobacco and tomato plants. The first-mentioned mosaic produces a severe lesion or "stripe" on the stem of the tobacco plant and a mosaic mottle on the tomato, while true tomato stripe acts in the reverse manner and produces severe lesions or stripes on the tomato stem and a mosaic mottle only on tobacco. In the case of some viruses certain particular plant species are useful as differential hosts, and this is exemplified by *Physalis* which has been used by James Johnson (1926 *a*) to distinguish a certain tobacco mosaic. Finally the question of local lesions as a diagnostic character must be touched upon. *Nicotiana glutinosa* is particularly useful in this respect in that certain viruses of the "tobacco mosaic" type show local lesions only and do not become systemic, while other somewhat similar viruses not only become systemic but are often fatal to the plant. Kunkel (1932), working with aucuba or yellow tomato mosaic obtained from England, has found that this virus produces necrotic local lesions on certain species and varieties of *Nicotiana* and chlorotic local lesions on others. Kunkel differentiates tomato aucuba mosaic from ordinary tobacco mosaic by its capacity to produce local lesions in tomato and in certain species and varieties of *Nicotiana*.

From this short discussion it will be seen that plant virus differentiation can be achieved to a certain degree by the various methods of study outlined in this review, and it is probably only a matter of waiting until sufficient information can be collected concerning each separate virus. One can then look forward to a time when a standard series of tests will be evolved to which each apparently new virus can be submitted and its reactions compared with those of known virus entities.

IV. THE POTATO MOSAIC GROUP.

With the possible exception of the mosaic diseases affecting the tobacco and tomato plants it is in the potato mosaic group that the worst confusion exists at the present time. In the ensuing section therefore, after reviewing certain papers on the subject, the writer endeavours to present a clear summary of what he considers is the present position regarding potato mosaic diseases. It should first be stated that certain potato viruses are not dealt with in this section; these are potato leaf-roll, spindle tuber, leaf-rolling mosaic and one or two others less important which do not occur in the British Isles. The potato viruses which are considered here are those contained in the diseases referred to as "mosaic," "crinkle" and "streak."

Salaman (1930) gives a description of the potato disease crinkle as defined by Murphy and others, which he has renamed Crinkle "*A*." In transmission experiments with this disease, it was found that in those cases where grafting to healthy potato had produced a crinkling, transmission by needle produced a mosaic only. The possible cause of the distinction between needle inoculation and grafting is discussed and the suggestion is made that it rests primarily on the minuteness of the original effective dose attained by needle inoculation. These results and others of Salaman's are now explained by the discovery that the crinkle disease is due to a virus complex and not to a single virus entity.

An interesting potato disease of the crinkle type has been described (Salaman and Le Pelley, 1930) which has been named Paracrinkle to distinguish it from Murphy's crinkle (= crinkle "*A*"), and Murphy has suggested that it may be the same disease as that known as leaf-rolling mosaic in America with the possible addition of another virus in the case of the latter disease. Paracrinkle was found latent in the potato variety King Edward, it is also carried without symptoms by the potato President, but when transmitted to Arran Victory it produces symptoms of a pronounced and violent crinkle. This virus appears to be non-transmissible by needle inoculation and the insect vectors are not known. Salaman and Le Pelley find that *Datura* can be infected with paracrinkle by grafting scions of infected and visibly diseased plants, and equally by grafting scions of "carriers." The symptoms induced are characteristic and distinct. It is stated that double grafts made by grafting *Datura*, with and without leaves attached, to Arran Victory and a paracrinkle scion to the *Datura*, demonstrate that passage through the tissue of a solid *Datura* stem, 2 inches long, has no effect on the virus, but that the presence of the leaves and the mixture of their metabolic products in the stem tissue with the virus passing through it destroys the virus. It is not made clear why, if the *Datura* plant can be normally infected with paracrinkle by grafting, the paracrinkle virus should be destroyed by the *Datura* scion in the double graft.

In a more recent paper on the analysis and synthesis of some diseases of the mosaic type, Salaman (1932) suggests the existence of a third virus "*Z*," which acting alone has a very limited pathogenicity, but acting in unison with either *X* or *Y*, or both, it is stated to bring about the diseases known as Crinkle "*A*" and Paracrinkle. Some interesting theories on the "linking" of virus entities, the phenomenon of auto-infection and the artificial production of symptomless virus "carriers" in potatoes are also introduced. In an analysis of necrotic virus diseases of the potato, Salaman and Bawden (1932) give a summary of the literature on streak, from which it appears that two distinct clinical states can be isolated. One of these is that described by Orton and commonly known as stipple streak or leaf-drop streak and later designated, on the grounds of its histology, as acropetal necrosis. The other, known as top-necrosis, has been described by Quanjer on the basis of its histopathology as acronecrosis. Acronecrosis or top-necrosis has been shown to be divisible into at least four distinct groups based on their varietal reaction, designated top-necrosis *X*, *A*, *B*, *C*. The first three are alike in that when they do produce a top-necrosis in any given variety, it is unaccompanied by any mosaic symptoms. Top-necrosis *C*, on the other hand, differs clinically by the fact that necrotic and mosaic symptoms occur together.

Recent work of the writer (1931 *c*) and others (Koch, 1931) has shown that most potato mosaic diseases are complexes containing more than one mosaic virus. More recently Murphy and M'Kay (1932) have published a paper in which they also deal with the composite nature of a potato virus disease, *i.e.* crinkle; in addition, Murphy (1932) has published a separate paper criticising the work of the writer in regard to those complexes. The remainder of this section then will be devoted to an attempt to reconcile the work of Murphy and M'Kay with that of the writer and to

clear up some of the confusion at present existing with regard to this group of diseases. The writer has shown that two viruses, provisionally designated as X and Y, occur frequently in combination in potato diseases of the mosaic, crinkle, streak type (1931 c); one of these two, the Y virus, is transmitted by the aphis *Myzus persicae*, the other virus, X, is not so carried. These viruses have been described in terms of their reaction upon the tobacco plant, on which the X virus produces concentric rings (see Plate I) while the Y virus produces a darkening of the tissue along the veins; a third virus was also considered to be present in crinkle and streak. These two viruses then, X and Y, have been found to be present, either separately, causing independent diseases, or in combination in a very large number of cases, while the Y virus which is so easily carried by the aphis *Myzus persicae* is thought to be—in England at any rate—the most destructive of all potato virus diseases, partly owing to its extremely common occurrence and partly to its lethal effect of "leaf-drop" on so many varieties of potato.

Murphy's criticisms of the writer's work seem partly based on a misapprehension of the use of the words "streak," "crinkle" and "mosaic." These words have been used by the latter to designate a set of *symptoms*, while Murphy uses them to designate a specific virus. Murphy and M'Kay introduce a new potato virus which they have named A; this virus is apparently confined to the potato and so does not show upon the various "indicator" plants used by the writer; virus A is also apparently not sap-transmissible. They consider A to be a necessary component virus of crinkle, the other component being Murphy's simple mosaic, which is probably the same virus as the writer's X. Murphy considers the Y virus which so frequently occurs in crinkle to be incidental or a contaminant and not a necessary component of the true crinkle disease. The writer would agree with Murphy that there exists another virus—the virus A—which will not infect tobacco and *Datura* and has already suggested as much (1931 c). He would also agree that there exists an independent virus occurring as Up-to-Date streak. As regards the X virus, or X-type virus, this is exceedingly common and is found in the majority of potato mosaic diseases. In Plate I are illustrated nineteen strains of the X virus isolated from various sources. It is necessary to say a word here about the X virus: in the large number of examples of this virus isolated by the writer, it has been found that, although they resemble each other in their main characteristics, *i.e.* in certain physical properties, in their non-transmissibility by the aphis and in the fact that addition of a standard strain of Y to any one of these X strains produces in tobacco an identical necrotic disease, nevertheless there are slight differences which seem sufficient to allow of the suggestion that these various X-types are variants of one virus and are probably not permanent mutations. Thus as regards the symptoms, the rings may be large or small, single or concentric, and even half double rings, and again it has been found that, while the "standard" X virus will not infect *Petunia* sp. (Smith, 1931 c) or only does so with great difficulty, other strains will infect the same plant very easily. This variation in the X-type of virus may perhaps be likened to the modifications occurring in the bacteriophage called "random variability" and "adaptability" by Prausnitz (1927), who concludes that these phenomena are strong evidence that the bacteriophage is a living entity. It must be

pointed out that when the X viruses were first transmitted to the tobacco plant they did not in the majority of cases produce the necrotic concentric rings. The most common symptom expression was a vague mosaic mottling with associated darkening of the green tissue; continued passage through tobacco, however, sooner or later always produced the necrotic rings. It was sometimes as long as one or two years in the tobacco plant before the symptom expression changed to the necrotic rings, but the change when it does occur is a sudden one. Murphy (1932) considers that the virus X after prolonged sojourn in tobacco undergoes considerable change, is no longer a potato virus and should not be treated as such; the writer would agree with this in so far as to admit that there is almost certainly an increase in virulence which appears to coincíde with the change over to the necrotic ring symptom expression.

As regards paracrinkle (Salaman and Le Pelley, 1930), this is an unusually interesting virus. It appears to be transmissible only by grafting and to be confined in nature to the potato variety King Edward. In the writer's opinion this virus has been seen only upon the potato plant and has not yet been transmitted to other members of the Solanaceae.

In conclusion, then, the position seems to be that there are at least five, and possibly more, distinct potato mosaic viruses, excluding those not dealt with here (p. 164); these are the writer's X and Y, the A virus of Murphy and M'Kay, Up-to-Date streak and paracrinkle. It is almost certainly the case that X and Y occur in conjunction with the other viruses, but some of these constituents of a disease may in a sense be incidental, in so far as their symptoms are obscured or overshadowed by those of the other viruses which cause the visible disease. Murphy states that his crinkle definitely consists of a combination of virus A and simple mosaic (= X) and that the disease cannot be otherwise produced; on the other hand the writer has frequently observed a crinkle disease in President induced by inoculation the previous season with the Y virus alone. Here again Murphy presumably refers to his specific crinkle disease, while the writer refers to crinkle-like symptoms.

As regards the insect vectors of these five potato mosaic viruses, there appears to be definite evidence in the case of only one of them. This is virus Y, which is transmitted with great efficiency by the aphis *Myzus persicae*; it seems clear that the X-type of virus is not aphis-transmitted (Smith, 1931 c), and it is difficult to explain the universal occurrence of this latter virus. Failing incrimination of some of the more obscure insect and allied fauna of the potato plant which have not yet been thoroughly tested, it may be necessary to consider some methods of virus transmission other than by the agency of insects to account for the dissemination of some of the potato mosaic viruses.

With regard to the insect transmission of the X-type potato mosaic the following observations·are relevant. The writer has planted young tobacco and *Datura* plants in the field among potato plants which were known to be affected with both X and Y. After some weeks it was found that numbers of the tobacco plants had contracted the Y disease only[1]; the *Datura* plants, resistant to Y, remained healthy.

[1] At the end of September two tobacco plants were found to have contracted the X + Y complex.

Similarly inspection was made of a field in the vicinity of Cambridge where tobacco was being grown commercially. While the crop was heavily infected with the Y virus, brought no doubt by *Myzus persicae* from nearby potato fields, there was no sign of the X-type of disease on any of the tobacco plants.

V. SUMMARY.

The chief methods of approach to the plant virus problem are outlined. These are dealt with in sections, the first being a study of various physical properties. The effect of heat on a number of plant viruses is given, the thermal death-points varying from 80 to 90° C. for that of tobacco mosaic to 42° C. for that of tomato spotted wilt. The reactions of various chemicals, including certain enzymes, with viruses are also studied. As regards dilution, it is found that while tobacco mosaic viruses will withstand dilutions of 1 in 10^4, the potato mosaic group are easily inactivated by quite low dilution. The various methods of purifying and concentrating virus solutions are discussed; these consist of concentration by means of Elford's membranes, precipitation by chemical methods, and by high-speed centrifuging. The filterability of a number of viruses is shown and it is found that certain viruses are not filterable by candle methods; in one case it has been found possible to filter a virus by means of collodion membranes when candle filtration was not possible. As regards longevity *in vitro*, tobacco mosaic virus will retain infective power for a year or more, while a certain potato virus, mosaic of cucumber and tomato spotted wilt become inactivated after ageing for varying periods of hours only. It is found that ultra-violet rays inactivate the virus of tobacco mosaic, when sufficiently free from contaminating material, in less than 15 sec.

In the next section the symptomatology of virus-affected plants is discussed under two heads, *i.e.* the appearance of diseased plants and their pathological histology; under the latter heading views concerning the nature of the intracellular inclusions, known as X-bodies, are put forward.

In the third section the various methods of virus transmission are reviewed. These are classed under the following heads: grafting, inoculation, insect agency, and transmission by seed and pollen. The relationship of the various plant viruses to these modes of transmission is indicated.

In the fourth section it is shown that so far no plant virus has been cultivated *in vitro*, and in addition some supposed causal organisms are discussed.

The movement of virus within the plant host is dealt with in the fifth section, and it is found that translocation of the virus of aucuba mosaic in the tomato plant takes place in the living ground tissue and in the phloem. Normally the virus agent does not enter the water stream of the plants, but, if introduced experimentally, it is carried round in the xylem vessels, from which it cannot escape unless the vessels are mechanically injured.

In the ensuing two sections the subjects of the metabolism of virus-affected plants and photography of viruses by ultra-violet light are dealt with. The chemical changes which take place within virus-affected plants are outlined.

Some other aspects of the behaviour of plants affected with virus diseases are next considered. These are dealt with under the headings of "recovery," "'carrying' power," "resistance" and "immunity." There are several authentic cases of the apparent recovery of plants affected with virus diseases; in all those cases the plants in question were susceptible to reinfection with the same virus. The best known examples of virus carriers are given and the suggestion is made that alteration in the environment, and virulence of the virus carrier, may influence this phenomenon in the potato plant. Some degree of resistance to a virus may occur among individuals of plant species which are normally susceptible to that virus. Resistance to mosaic occurs in many varieties of sugar-cane, and to curly top in certain individual beet plants. A new variety of potato, "Katahdin," apparently resistant to the writer's " *Y* " virus, is mentioned. Plants do not develop an immunity[1], but certain species of plants appear to be immune to a specific virus which affects other closely allied members of the same group. Finally in this section some papers on immunologic reactions of tobacco mosaic virus are dealt with. It is shown that separate antisera are produced in rabbits to normal sap from healthy tobacco plants and to virus sap from mosaic plants. Appropriate quantities of the antiserum to the virus sap of tobacco are capable of completely inactivating the virus sap.

As regards the electric charge of virus particles, it has been demonstrated that tomato mosaic virus, or the particles containing it, migrate to the anode in an electric field at pH readings of 5·3–8·5.

The application of the various methods of study to the differentiation of plant viruses is next discussed, and the importance of classifying the virus and not the disease it causes is emphasised.

Finally, some recent work on potato mosaic diseases is reviewed and an attempt made to give a clear statement of the present position as regards the potato mosaic group. An extensive bibliography of the literature on plant virus diseases is included.

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[1] See Price, page 178.

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APPENDIX.

After the MS. had gone to press, a number of papers relevant to this review have come to hand and these are listed at the end of this Appendix. It is only possible here to refer briefly to some of the more important facts. Johnson and Grant (1932) have examined certain physical properties of plant viruses when in different host species and have confirmed the view, already generally held, that the properties of a virus are inherent and do not vary with the species of host plant. Murphy and M'Kav (1932) have published a comparison of some European and American virus diseases of the potato, they consider that only leaf-roll, aucuba mosaic and inter-veinal mosaic are identical on the two continents; they state that rugose mosaic is not identical with crinkle. No mention is made, however, of the aphis-transmitted potato virus, " *Y*," described by the present writer, which is one of the commonest and most destructive of the potato viruses in England. Storey (1932) has published the complete account of his work, already referred to on p. 152, on the interesting fact that the ability in a leaf-hopper to transmit the virus of streak of maize is inherited as a simple dominant Mendelian factor, linked with sex. Kunkel (1932) describes two strains of "yellows" disease which are symptomatically similar and are both transmissible by the same insect vector, *Cicadula sexnotata*; the two viruses are differentiated by the fact that one strain, originating in California, is transmissible to celery while the other strain, from New York, is not transmissible to celery. This occurrence of two apparent strains of the same virus transmitted by the same insect is of interest in view of Storey's recent observation (1931) of two apparently distinct

maize diseases, symptomatically similar to streak of maize, but *not* transmissible by
the vector of this latter virus. Storey's suggestion that plant viruses are better
characterised by the insect vectors than by the plant hosts (1931) would therefore
not be applicable in the case of the two types of aster yellows described by Kunkel.
Holmes describes some new types of symptoms (1932 a) produced by "ordinary"
tobacco mosaic upon differential hosts. He considers that there are three kinds of
tendencies towards natural immunity to tobacco mosaic in Solanaceous plants.
These he refers to as a partial resistance—(a) to infection by the virus, (b) to increase
of virus, (c) to spread of virus. The same worker (1932 b) has studied the movement
of mosaic virus from primary lesions in *Nicotiana tabacum*. Holmes finds that the
average interval between time of inoculation and appearance of systemic symptoms
is shorter when the number of primary lesions is large than when the number of
primary lesions is small, and when the inoculation is near the base of a leaf than when
it is near the apex of a leaf. Price has published an interesting paper (1932) entitled
"Acquired immunity to ringspot in *Nicotiana*," in which a description is given of
tobacco plants which have been severely affected with the ringspot disease and which
recover and cannot be reinoculated with the virus. As all these recovered plants,
however, are still systemically infected with the active ringspot virus, they have not
acquired an immunity in the usual conception of that term. Alternative titles to
Price's paper would be—"Acquired immunity to the clinical picture of ringspot in
Nicotiana," or "An acquired tolerance by *Nicotiana* to a ringspot virus."

Finally instances of apparent cultivation *in vitro* of two animal viruses must be
quoted as being relevant to the general subject of viruses. These are firstly the
cultivation of vaccinia virus in a cell-free medium of ground kidney emulsion by
Eagles and Kordi (1932), and secondly the cultivation, according to Beach (1932),
of the virus of infectious laryngo-tracheitis of chickens in a medium, presumably
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EXPLANATION OF PLATE.

In this plate are illustrated strains of the *X* potato mosaic virus obtained from nineteen different sources; they are represented in terms of their reaction upon the tobacco plant, White Burley variety. In many of these cases the *X* virus was accompanied in the potato disease by the *Y* virus, which had to be eliminated. In the case of the crinkle plants it must be assumed that the *A* virus of Murphy and M'Kay was also present; this latter virus is apparently not transmissible to tobacco. The tobacco plants were inoculated from the following sources:

Fig. 1. Potato Up-to-Date carrying *X* and streak; this is the writer's standard strain of *X*.

Fig. 2. Potato Arran Victory showing mild mosaic.

Fig. 3. Another Arran Victory also with mosaic.

Fig. 4. Potato Myatt's Ashleaf showing severe crinkle.

Fig. 5. Another Myatt's Ashleaf with crinkle.

Fig. 6. Potato Arran Comrade with crinkle.

Fig. 7. Potato Up-to-Date from Rhodesia, sent as healthy.

Fig. 8. Potato Up-to-Date from farm plot, showing no symptoms.

Fig. 9. Potato President showing interveinal mosaic.

Fig. 10. Potato Majestic with faint mosaic mottling.

Fig. 11. Potato Arran Pilot showing no symptoms.

Fig. 12. Potato Arran Consul from farm plot, showing leaf-roll and crinkle.

Fig. 13. Potato President, plant in "curly dwarf" stage.

Fig. 14. Potato Great Scot, wilding.

Fig. 15. Potato Arran Banner, mosaic.

Fig. 16. The *X* virus in the form of half-rings; this appears to be due to attenuation of the virus.

Fig. 17. Potato Brownell Medium-Top from Tasmania, showing no symptoms in glasshouse.

Fig. 18. Potato Bismarck from Tasmania, sent as rugose mosaic, but showed no symptoms in glasshouse.

Fig. 19. Tobacco inoculated with two different strains of the *X* virus; note the rings of the standard *X* (as in Fig. 1) superimposed upon the other *X*, which is in its "mottle" form.

Fig. 20. The same *X* virus, shown in "mottle" form in Fig. 19, now itself changed over to the ring formation.

ON THE DISSOCIABILITY OF THE FUNDAMENTAL PROCESSES IN ONTOGENESIS

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(With Ten Text-figures.)

CONTENTS.

I. INTRODUCTION.

In the development of an animal embryo, proceeding normally under optimum conditions, the fundamental processes are seen as constituting a perfectly integrated whole. They fit in with each other in such a way that the final product comes into being by means of a precise co-operation of reactions and events. But it seems to be a very important, if perhaps insufficiently appreciated, fact, that these fundamental processes are not separable only in thought; that on the contrary they can be dissociated experimentally or thrown out of gear with one another. This conception of

out-of-gearishness still lacks a satisfactory name, but in the absence of better words, dissociability or disengagement will be used in what follows. It is already clear that embryonic growth can be stopped without abolishing embryonic respiration, and conversely, it is probable that growth or differentiation, under certain conditions, may proceed in the absence of the normal respiratory processes. There are many instances, again, where growth and differentiation are separable. It is as if either of these processes can be thrown out of gear at will, so that, although the mechanisms are still intact, one or the other of them is acting as "layshaft" or, in engineering terms, is "idling."

Since one of the principal aims of embryology must be to understand the means whereby in the normal embryo the fundamental processes are integrated, it is probably worth while to devote some attention to the cases in which this integration has been artificially broken down. Such cases are now fairly numerous, but do not appear to have been previously brought together. It is not claimed that the present review forms a complete survey or list of them—no doubt every embryologist would be able to add to their number from his own experience—but the mere reduction to order of a field of data where the individual constituents are not at first sight obviously related to one another, is in itself an important forward step.

Before proceeding farther it will be advisable to define more clearly the fundamental processes which are under consideration. For the present purpose, the classification shown in Table I will be adopted. It is, of course, crude and incomplete, but it will suffice where practical experimental facts are under discussion. For a more refined treatment the interesting papers of J. H. Woodger (1930, 1931) should be consulted.

Table I. *Chart to show the definition of the fundamental
processes discussed in this review.*

A. GROWTH: increase in spatial dimensions and in weight.	1. (Multiplicative: increase in number of nuclei.) 2. Multiplicative: increase in number of cells. 3. Intussusceptive: increase of size of cells. 4. Accretionary: increase in amount of non-living structural matter.

B. DIFFERENTIATION: increase in complexity and organisation.	1. Increase in number of kinds of cells:
	   (a) invisible. Determination of fates; segregation of potencies; loss of competence, etc.;
	   (b) visible. Histogenesis.
	 2. Increase in morphological heterogeneity; the assumption of form and pattern; organogenesis:
	   (a) individuative: due to the primary Individuation Field of the organism before any of its parts have become functional;
	   (b) corporative: due to the mutual interactions of functional parts.

C. METABOLISM: the chemical changes in the organism.	1. Respiration, *i.e.* oxidation of carbon compounds. 2. Fermentation or glycolysis: the non-oxidative catabolism of carbohydrate. 3. Catabolism of protein. 4. Catabolism of fat. 5. Characteristic chemical activity: pigment-formation, *e.g.*, or the synthesis of glycogen.

Notes to Table I.

(1) The grouping of the processes is difficult and perhaps necessarily arbitrary. Thus C 5 might possibly be placed in B; A 4 in C; and A 1 and A 2 in B.

Although increase in number of nuclei and increase in number of cells is not growth in the sense of size expansion, it is so intimately bound up with growth that it is placed in group A. Nuclear and cell division can occur without size expansion in many organisms, *e.g.* developing marine inverte- · brates in the cleavage stages, but even here there is usually some swelling of the system.

(2) The terms of A 2-4 are those recently adopted by Huxley (1932), but the restriction of "Multiplicative" to cell division, and "Intussusceptive" to cell-size increase, is here proposed for the first time.

(3) B 1 needs further comment:

(i) Increase in number of kinds of cells may be either reversible or non-reversible. The specialisation of the histological elements in a tissue such as the kidney (which may be reversed in rapidly growing explants) is non-determinative or reversible, but the original segregation of potencies which occurs early in development is probably not reversible. Thus the kidney cells can lose their differentiation but they cannot differentiate off into liver cells.

(ii) The notion of Competence has recently been defined, and the concept of Segregation discussed, by Waddington (1932 *a*). The term "Chemodifferentiation," which was introduced by Huxley (1924) to cover all determinative processes, may perhaps now be laid aside in favour of better, because more empirical and less doctrinaire, expressions. It invites confusion with the "chemical differentiation" of Murray (1926), which applies to the changing distribution of chemical substances in the embryo, and which has more logical justification.

(iii) Huxley (1932) now introduces two further terms—"Histodifferentiation" and "Auxanodifferentiation." He distinguishes two periods in the growth of a part, firstly, a laying down of general form, accompanied by rapid morphological and histological change; secondly, a cessation of histological change, and a reduction of the form change to "quantitative alterations in the proportions of the definitive structural plan." For these two periods he suggests the terms "Histodifferentiation" and "Auxanodifferentiation" respectively. I am unable to see in what way this latter process differs from Growth, since growth, defined as increase in spatial dimensions, need not necessarily take place equally in all of them. The valuable investigations of Huxley on heterogony show, indeed, that it hardly ever does. And reference may be made to those directed cell streams, the existence of which is so important for early differentiation, and the nature of which is so obscure. Again, if we define differentiation (as in B) as increase of complexity and organisation, "quantitative alterations in proportions" of a fixed plan, can hardly be included in it. Such alterations amount to deformations and surely appertain to the sphere of relative Growth. "Histodifferentiation" appears to be covered by B 1 (*b*) and B 2 (*a*).

(iv) B 1 (*b*) is termed by Woodger (1930) "elaboration." "Differentiation" he defines as "a process whereby two (or more) different parts come into being by the separation unequally between them of something previously present in one part (or whole)." This definition would include all of B 1 but hardly all of B 2.

(4) B 2 (*a*) is given the term "Individuative" after the important concept of "Individuation Field" introduced by Waddington (1933). It largely corresponds with the Tendency towards "Ganzheit" discussed by Bertalanffy (1928, 1929)—"Die organische Gestalt strebt nach einem Maximum von Gestaltheit"—but arises more directly out of experimentation.

B 2 (*b*) is given the term "Corporative" since in the late stages of development (the secondary or functional period) the various parts of the organism react upon one another as members of a corporation.

(5) It may be noted that processes C 1-4 are all energy-providing processes, whereas C 5 need not be, and may be the reverse.

II. DISENGAGEMENT OF GROWTH AND DIFFERENTIATION.

(1) *Anidian embryos: growth without differentiation.* The case of the "anidian" deviation in the development of birds is of considerable interest. The anomalous formation of the blastoderm without the appearance of primitive streak or other embryonic structures, *i.e.* the growth of the flat mass of cells upon the yolk without embryonic differentiation or organisation, was already known to Isidore Geoffroy de St Hilaire, but not much studied until the sixties of the last century. Panum (1860) described chick "embryos" with well-developed blastoderms but no primi-

tive streak, and two years later Broca (1862) reported that the frequency of this disassociation of growth and organisation was much increased if some time were allowed to elapse (*e.g.* three weeks) between laying and the beginning of incubation, the fertile eggs being kept at room temperature[1]. Then Dareste (1877), in his classical work on teratology, observed that temperatures higher or lower than normal, maintained during the first day or two of incubation, would lead to a high percentage of blastoderms without embryos. He drew attention to the difficulty of distinguishing between true anidians and dead blastoderms which had possessed the beginnings of a primitive streak but from which this had disintegrated, and was inclined to suppose that a good many of Panum's anidians were of this character. Dareste showed that the absence of embryonic structures could co-exist with a considerable development of the *area vasculosa*. Later work of a descriptive and morphological kind has been done by Rabaud (1899), Tur (1929) and Grodzinski (1931), but more important for the present line of thought is the fact that Edwards (1902) found he could obtain no less than 63·4 per cent. of anidian blastoderms by incubating hens' eggs at temperatures between 20 and 27° C.

It seems clear, therefore, that under certain abnormal conditions, the usual relations between growth and differentiation, *i.e.* the coming-into-being of new organised structures and spatial arrangements of cells, can be thrown out of order. The anidian is a result of disengagement between the processes of growth and differentiation. The metabolic peculiarities of anidian blastoderms would well repay investigation; here a beginning has been made (Needham, 1932*a*) by a determination of respiratory quotient.

(2) *Experimental dwarfism: differentiation without growth.* Another remarkable instance of the disengagement of growth and differentiation is afforded by the work of Hoadley (1929), who transplanted embryonic organs of the chick to the chorioallantoic membrane and allowed them to develop there until they had reached a degree of differentiation equivalent to that of the controls. Then by making and weighing wax models he ascertained the relative weights, and always found that the controls were much heavier. But there was a direct relation with age, for the younger the transplant at the time of transplantation, the smaller the eventual fully differentiated organ, as is shown in Table II.

Table II.

Organ	At the time of transplantation		After 8 days, size of control: size of transplant 1 : *x*
	Embryo age (hours)	No. of somites	
Spinal cord	48	28	1 : 0·353
Eye	48	28	1 : 0·219
Eye	35	14	1 : 0·098
Eye	20	0	1 : 0·0136
Eye	4	0	1 : 0·00075

[1] It is of interest that the percentage viability of fertile eggs held at room temperature falls off to nil, after about a month, along a regular curve (Moran, 1925).

Thus in all cases the growth of the graft was very much inferior to that of the control in its normal situation, although differentiation, as judged by the degree of histological development and cellular elaboration, was the same. There seems no obvious mechanical reason why this suppression of growth occurs, for the transplanted fragment becomes surrounded by highly vascular tissue surrounded on one side by the epithelial margin of the allantois and on the other by the repaired chorionic epithelium. Capillaries of the host membrane ramify freely in the neighbourhood of the transplant, the cells of which should have no difficulty in obtaining from them the nutritive building stones required for growth.

Hoadley explains these results by the assumption of a lag period immediately after transplantation which would be felt more acutely by the youngest and therefore smallest fragments. During this time cell division would be suppressed but histogenesis would be proceeding. It remains obscure, however, why the cell division should be most fully suppressed in the case of the youngest grafts. "Differentiation," says Hoadley, "is not primarily dependent upon any mechanism involved in specific cell divisions, but to a large extent takes place independently of these. Inasmuch as this is true, and inasmuch as typical development depends not only on the subordinate differentiation of the constituent parts of the embryo, but also on the spatial relations between them and their size, the truth of the following statement is evident—'typical development is the result of the usual balance between morphogenetic (form producing) and histogenetic (cell differentiating) processes.'"

The derangement of this balance appears again in other instances. Thus Waddington (1932 a), in the course of extensive studies of the organiser phenomena exhibited by chick embryos when cultivated *in vitro*, observed that the mere development of the blastoderm outside the egg gave rise to a considerable disengagement of the growth and differentiation processes. Both were slowed, but the former more than the latter. For the formation of one somite under normal conditions *in vivo* between the stages of 2 and 27 somites, 1·04 hours are required, but *in vitro* the time taken was found to be between 2·44 and 1·15 hours, with an average at 1·65 hours. This reduction in the rapidity of somite formation was approximately constant whatever the age of the embryo at the beginning. On the other hand the growth (measured as increase in embryo length) was very much slowed. "The stage of differentiation attained by an embryo," writes Waddington, "is very largely independent of the absolute size, and if a blastoderm is explanted at an early age, and thus is affected by the slowing of the growth rate for the whole of its life, an embryo may be formed which, compared with the normal, is very much too small for its stage of differentiation." Thus an embryo of 19 somites, which *in vivo* is 5·3 mm. long, may be *in vitro* only 2 mm. long[1].

There are many other reports of experimental dwarfism in the literature, and many of these might be taken to provide evidence of the disengageability of growth and differentiation. Mention of one recent one only will be made. Bohn and

[1] Homozygous Creeper embryos, recently studied by Landauer (1932), manifest a similar preferential retardation of growth; morphogenesis being little impaired. In this case abundance of nutriment is certainly present.

Drzwina (1931) subjected the developing eggs of the sea-urchin (*Strongylocentrotus*) to $N/20$ and $N/10$ solutions of phenyldimethylbetaine in sea water. It was found that if the treatment was begun at the gastrula stage, there was no stop to differentiation, and normal plutei were formed, but less than half the size of the controls. The betaine plutei provided a striking instance of a derangement of gearing as between differentiation and growth.

(3) *Nuclear division without cell division, and differentiation without either.* The experiments of Hoadley, referred to in the preceding section, were to some extent inspired by the numerous earlier works, dating from about the beginning of this century, in which it was shown that morphological differentiation is possible without either cell division or nuclear division.

Chabry (1888) first succeeded in suppressing protoplasmic segmentation but not nuclear segmentation by subjecting ascidian eggs to pressure, and Loeb (1892, 1895), using hypertonic sea waters, found that a segmentation of the nucleus might take place without any cell division, with the production of multinuclear single cells or multinuclear blastomeres. The experiments were carried much farther by Norman (1896) who succeeded in getting reproducible results by the aid of abnormal concentrations of magnesium chloride. Multiple mitosis regularly took place when *Arbacia* eggs were made to develop in sea water containing 2 per cent. $MgCl_2$. After a stay of some hours in solutions containing amounts of magnesium of this order, from 11 to 18 nuclei could be counted within the fertilised but still quite undivided cell, and a similar suppression of cell division while the nuclei continued to divide, was found when fish eggs (*Ctenolabrus*) were treated similarly. In the case of *Ctenolabrus* high temperatures, *e.g.* 30°, were more effective, and blastodiscs containing many nuclei but without a trace of cell division could easily be obtained. Later Morgan (1899) criticised some of Norman's conclusions, but for the most part confirmed the possible dislocation of the phenomena of nuclear and cytoplasmic cleavage. Work of his own, moreover (1900), on the effect of strychnine on echinoderm eggs, brought to light a similar dislocation. And Driesch (1899) found other instances in which abnormal temperatures produced polynuclear undifferentiated masses from fertilised eggs. Barta (1926), too, could encourage the production of giant polynuclear cells in explanted rabbit lymph nodes by subjecting them to conditions of poor oxygenation[1].

In some forms, especially the arthropods, nuclear division occurs naturally without cell division in the ordinary course of development. Thus in the classical case of *Astacus*, the zygote nucleus divides into some twenty descendant nuclei which migrate towards the periphery of the egg. The egg then divides into a series of radially arranged pillars or yolk pyramids each having a nucleus at its peripheral end. The pyramidal blastomeres do not, however, persist, for their intervening planes break down in the central part of the egg, and give a layer of cells surrounding an internal mass of yolk. And a similar state of affairs is found in all insects, arachnids, and crustaceans.

So far we have only described cases in which differentiation was out of play, and

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[1] See also the admirable work of Chalkley (1931) on polynucleate amoebae.

cell division and nuclear division (defined for the present purpose as two components of growth) were disengaged. It would seem very unlikely, *a priori*, that we could have a differentiation proceeding in the absence of either cell division or nuclear division. Yet this extraordinary state of affairs was described by F. R. Lillie (1902) in a classical paper on the egg of the annelid *Chaetopterus*. After a short exposure to abnormal concentrations of potassium in sea water, the eggs, without dividing into cells, passed through certain well-defined phases of differentiation, the yolk accumulating in a dense mass in the interior, and the peripheral cytoplasm becoming vacuolated and ciliated. "The ciliated ectoplasm and the yolk-laden endoplasm," said Lillie, "are analogous to the ectoderm and endoderm of the
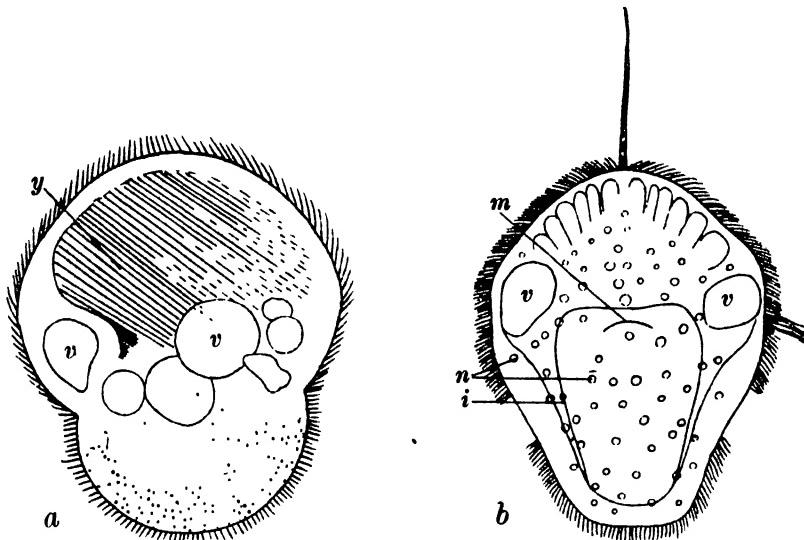
Fig. 1. Differentiation without cleavage in the egg of *Chaetopterus* (rearranged from Lillie). To the left: a "ciliated object" developed from an unfertilised egg (after 24 hours). Treatment: in 95 parts of sea water plus 5 parts of 2½N KCl for 1 hour. To the right: a normal trochophore 24 hours old (same magnification). Reference letters: *i*, intestine; *m*, mouth; *n*, nucleus; *v*, vacuole; *y*, yolk.

trochophore, and the phases of differentiation resemble some of the normal processes, though the resulting object can by no stretch of the term be properly called a trochophore." Both in fertilised and unfertilised eggs could the phenomenon be demonstrated, and the action of the potassium ion was therefore in the first case to suppress certain events which arise normally, in the second case to induce certain events which do not normally occur.

The subject is of such interest that two of Lillie's illustrations are here reproduced. Fig. 1 *a* shows a well-developed ciliated object from his potassium cultures, Fig. 1 *b* a normal trochophore. The ciliated object is radially symmetrical round its long axis, and is divided by a constriction into a smaller and larger hemisphere. The yolk is aggregated in a dense mass in the latter, and a row of large vacuoles completely surrounds the body near the constriction; the protoplasm of the smaller

hemisphere is very dense and granular; the cilia were strong, active, and regularly placed. No cell structure could be detected in the living object. It presents, as Lillie said, "an undeniable resemblance to a trochophore; if the smaller hemisphere be compared to the pre-trochal, and the larger to the post-trochal region, the large vacuoles occupy approximately the position of the prototroch. A similar girdle of vacuoles is found in this position in the trochophore. The aggregation of yolk is in a similar position to the gut of the trochophore." And on sectioning it was clear that instead of the hundreds of nuclei visible in the normal trochophore, only one large nucleus was contained in such a ciliated object.

In a later paper Lillie (1906) reported that certain other treatments besides abnormal potassium-ion concentrations would produce the same result, *i.e.* letting the eggs remain abnormally long in ordinary sea water before fertilisation, or exposing them to abnormal temperatures after fertilisation. Moreover, the unsegmented eggs undergoing differentiation might be either mononuclear or polynuclear. Unfertilised eggs treated with KCl would be of the former type, fertilised eggs would be of the latter if they had undergone polyspermy or if they had shown an original cleavage followed by fusion of the blastomeres. It was found that the polynuclear unsegmented eggs differentiated into the "ciliated object" condition just as rapidly as normal eggs produced trochophores, whereas the mononuclear eggs took a considerably longer time to develop.

Chaetopterus did not long remain the only animal in which differentiation without cleavage had been observed, for Treadwell (1902) described it in another annelid, *Podarke*, and Scott (1906) found a particularly striking case in yet another, *Amphitrite*. Development without cleavage, under Scott's conditions of parthenogenesis, took the form of nuclear divisions, the early differentiation of a layer of ectoplasm, the growth of cilia, the appearance of vacuoles that are found in the fertilised egg of the same age, the development of brownish pigment, amoeboid movements of cytoplasm, and change in the general shape of the egg. The apical tuft of cilia characteristic of the normal trochophore was always absent, however, from these "ciliated objects" of *Amphitrite*, as also from those of *Chaetopterus*. Certain curious experiments of Bastian on rotifer eggs could not be explained, according to Lillie, except upon the supposition that differentiation without cleavage was a possibility there also.

Such considerations lead naturally on to a discussion of the relations between cell size and cleavage. Thus Henrici's (1921, 1923) interesting experiments on bacteria showed that in fresh media some cause operates favourable to increase of cell material but inhibitory to cell division, leading the majority of cells to attain an abnormal size before splitting occurs. At the end of the "lag period" of growth, for instance, the average length of *Bacillus megatherium* was six times that of the cells at the time of inoculation. Similar results have been obtained by Wilson (1926) on other bacteria, and Stephenson (1930) has reviewed the literature of the subject. But it would take us too far from the point at issue to deal adequately with the relation of cell size to cleavage, and a reference to a recent review (van Cleave, 1932) must suffice.

Much the most interesting recent contribution to this question from the embryological point of view is the work of Bhatia (1931) on the muscle cells of the developing

trout. Bhatia showed that throughout the free-swimming yolk-sac period after hatching, the growth of the muscle cells is entirely due to growth in size, and no cell divisions take place. But as soon as the larva begins feeding and the yolk has all been used, cell multiplication begins; the increase in cell size alone cannot now keep pace with body growth. These phenomena call to mind the statement of Wright (1926) and others, that avian yolk inhibits mitosis in explanted cells.

On the general question of differentiation without cleavage, the conditions in Protozoa may be remembered. An extraordinarily high degree of differentiation and morphological complication may exist in unicellular organisms (cf. Calkins, 1926, and footnote 3 on p. 208 below).

(4) *Differential action of hormones and amino-acids on differentiation and growth.* The endocrine system affords a powerful instrument for the disengagement of growth and differentiation, as was first realised by Gudernatsch (1912) in his classical work on the thyroid control of amphibian metamorphosis. This is so well known, and has so often been summarised (*e.g.* Gudernatsch, 1926, 1929) that it is unnecessary to recapitulate it here. "Wir haben es also hier mit Differen-zierung ohne Wachstum zu tun," said Gudernatsch (1929), reviewing his earlier work, "Wir wissen, dass in der Normalentwicklung des Individuums zwei Prozesse parallel laufen, Wachstum und Differenzierung. Im Kaulquappenversuch ist es uns zum ersten Male gelungen, diese Prozesse voneinander zu isolieren." And, indeed, the premature metamorphosis induced by thyroxin affords the standard example of the disengagement of two fundamental processes[1].

At the present time attempts are being made to discover a differential action of the amino-acids on growth and organisation. Hoffman and Gudernatsch (1931) have found that it is possible to rear tadpoles to metamorphosis on a diet in which a single amino-acid is the sole source of nitrogen; carbohydrates, fats and vitamins being given in the form of a basal food. It has thus appeared that the amino-acids differ in their effects on growth and differentiation. Arginine permitted growth but practi-cally no differentiation, as did cystine, lysine and phenylalanine. Tyrosine and tryptophane, on the contrary, led to a favouring of differentiation rather than growth, and produced small well-developed animals. In certain cases both growth and differentiation were suppressed, the tadpoles simply continuing without much change, *e.g.* leucine and alanine. Certain amino-acids, such as histidine and aspartic acid, hardly permitted even of maintenance. Di-iodo-tyrosine brought on the usual early metamorphosis, but this could be retarded, it was found, if a typical "growth acid" were given at the same time. Gudernatsch and Hoffman (1931 *a*, 1931 *b*) have since made a preliminary report concerning the possible permutations of the amino-acids, but none of this work has yet been published in full. Experiments of a some-what similar nature have been reported by Pincherle and Gelli (1931).

(5) *Disengagement of determination from differentiation and growth.* The term differentiation must be used to cover, not only the actual processes of histogenetic and organogenetic elaboration which come first to mind, but also those obscure

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[1] But see the critical discussion of Huxley (1932, p. 182). The general argument of this sub-section is not affected by the qualifications there laid down.

determinative processes which decide the fate of embryonic rudiments and allot them their subsequent spatio-temporal paths. The determinative factor in differentiation can, it seems, be disengaged from the growth process just as much as the other factors can. The particular points in ontogenesis at which "chemodifferentiation" occurs; the particular times at which groups of competent cells suffer their destined restriction of potency, form a constellation of characters which are at least constant for a given animal species. But the interesting work of Twitty (1928) who studied the development of the cilia on the skin of amphibian embryos shows that a determinative process can be shifted in time experimentally, and thus thrown out of gear with the growth processes.

Twitty found that the polarity of the ciliary cells is determined during the closure of the neural folds, for the cilia of ectodermal grafts rotated through 180° before that
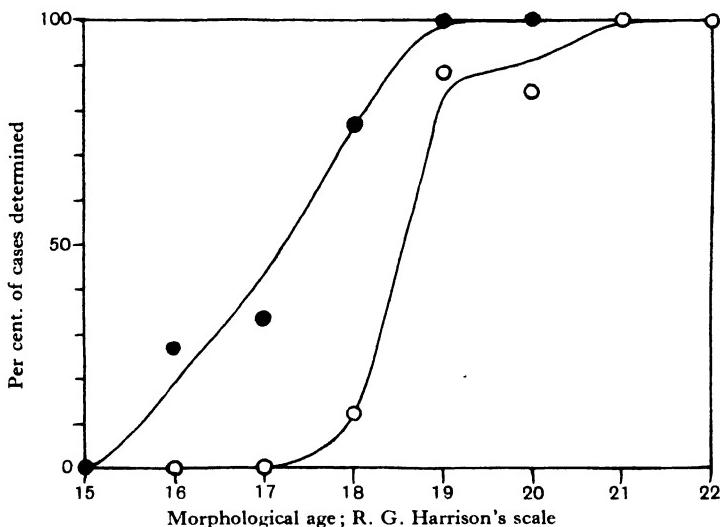
Fig. 2. Determination of ciliary polarity in *Amblystoma* (Twitty). Embryos kept at 15°, ○ ; at 7°, ● ; before the operation.

stage beat in the same direction as the cilia of the adjacent host ectoderm (*Ortsgemäss*), while ciliary grafts transplanted and rotated later retained their original direction of waving (*Herkunftsgemäss*). The time of determination, it was interesting to note, was not identical with that of the morphological determination of the ectoderm. Now in *Amblystoma* embryos allowed to develop at low temperatures, Twitty found that the ciliary polarity appeared at a much earlier stage. Evidently the determinative process had been disengaged both from the growth processes and also from the visible differentiation processes (which were all proceeding normally except for the retardation due to low temperature). As Fig. 2 shows, the percentage of transplantations showing determined ciliary polarity rose to its maximum earlier relatively to morphological age in the cooled embryos than in those maintained at a normal temperature. It would not be illegitimate to postulate a reaction inhibitory

to the determination process; such a reaction might have a temperature character-
istic higher than that of development as a whole[1]. No doubt future investigations
will give us the temperature characteristics of all the processes of chemodifferentia-
tion, segregation of potencies, loss of competence, etc., which at present are so far
from physiological analysis.

(6) *Genetic shift and re-engagement of differentiation and growth.* Independence
of growth and differentiation does not require experimental interference for its
demonstration, but manifests itself naturally in less spectacular ways. The genetic
races of rabbits which differ considerably in size are a case in point. At birth the
large-sized race is twice as heavy as the small-sized race, and at the adult stage three
times as heavy. Painter (1928) observed that equally differentiated 12-day embryos
showed the characteristic weight differences, and thought that the effect might be of
endocrine origin, but Castle and Gregory (1929) were later able to trace it back as
far as the morula stage. The large-sized race showed consistently more rapid cell
multiplication and size increase during the embryonic period, with unaltered
differentiation rate. There is no difference in egg size, but the large-sized race grows
more quickly. Thus at 48 hours from fertilisation the large-sized race has 22 blasto-
meres, the small-sized only 14. At 100 hours the diameter of the blastodermic
vesicle is, in the large-sized race, $31 \cdot 4\mu$, in the small-sized race only $16 \cdot 3\mu$. Between
birth and puberty, according to Robb (1929), there is no difference in growth rate,
but after puberty the small-sized race again falls behind.

Recently, similar investigations have been going on with regard to the differ-
ences between birds of different body size. Kaufman (1926, 1930c) first found that
the log. weight/age curves of hen and pigeon during embryonic life ran closely
together, and that the Schmalhausen 'growth constants (see Needham, 1931,
section 2) were for the hen 346, for the pigeon 353. Thus between the 3rd and the
21st day of incubation the instantaneous percentage growth rate of the two birds was
identical, and could not account for the differences of body size. Even when the
growth rates of the individual organs were compared, the differences between the
two birds were but slight. On hatching, however, large differences supervened,
probably due to differences in diet. The size difference was thus thrown back to the
period before the 3rd day, and Kaufman (1930b) found that it lay in a difference in
the size of the cells themselves, so that even at the time of the appearance of the
primitive streak, the size difference was marked[2]. The primitive streak appears at
the same time in fowls and pigeons, but its length in the two birds is as $1 \cdot 2 : 1$. Hence
the volume, and probably the weight, would compare as approximately 2 to 1. In
this case, therefore, we have to deal, not with a difference in the integration of
growth and differentiation processes, but with a difference in the size attained by a

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[1] Or, if the determinative process itself could be thought of as sufficiently chemical to have a
temperature coefficient, its coefficient might be lower than that of development as a whole.

[2] On the 3rd day of development, the dimensions of a liver cell are, according to Kaufman (1929),
for the chick 974 cubic μ, for the pigeon 596 cubic μ. On the 11th day they are for the chick 1223
cubic μ, for the pigeon 940 cubic μ. And no significant difference exists as regards frequency of
mitosis in the embryonic development of the two birds. Moreover, Milone (1923) found the number
of cells present at equivalent morphological ages in the neural tube of birds, so different in size as the
the chick and the sparrow, to be the same.

cell before cleavage takes place. Kaufman (1930a) attributes the discordance between her results and the earlier ones of Levi (1921) to the fact that he drew his conclusions solely from linear surface measurements of blastoderms, without taking into account the bigger effect which consideration of the volumes involved shows to exist.

Kaufman's work was all concerned with inter-specific differences, but Byerly (1930) has studied the growth of embryos from a larger (2·77 kg.) and a smaller (2·01 kg.) race of hens. The weight difference was perceptible in embryos only from the 10th day onwards[1], but in eggs of the same weight from the two breeds the difference tended to disappear towards hatching time, owing, it is suggested, to the limitation of food supply. Relative growth rates in large and small races of pigeons have also been investigated recently by Riddle, Charles and Cauthen (1932), who find that a part of the racial size differences is certainly expressed by the weights at hatching:

	Adult weight gm.	Hatching weight gm.
Runt	565	14·0
Homer	398	14·7
Magpie	296	11·5
Tippler	278	11·3

But a large share of the ultimate size differences in these races is attained through differential rate of growth after hatching during the first three weeks[2].

It is clear that much further work is necessary before the phenogenesis of body size can be said to be understood. In some cases, however, it is already certain that a shifting of the gears between growth and differentiation comes into play, and that the two sets of processes may be integrated in a different manner both inter-racially and inter-specifically.

(7) *Mutual independence of growth, histogenesis and organogenesis.* The technique of tissue culture has afforded many new possibilities of studying the relation between growth and differentiation. It was early found (cf. Strangeways, 1924) that successful culturen of metazoan cells *in vitro* proceeded along one of two directions, either cell division took place side by side with much "outwandering" from the original explant, or cell division occurred only in the explant itself. The first of these processes led to no increase in differentiation, and was often called "unorganised growth," the second might involve far-reaching histogenesis and even organogenesis, and was called "organised growth." The unorganised growth of tissue cultures is now thought to involve a kind of dedifferentiation, though how far this is a regression to a non-functional or embryonic state is a question still unsettled.

The earlier workers supposed that histological differentiation *in vitro* could only take place if several types of cells were present to begin with, but the power of

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[1] From this it would follow that Castle and Gregory's explanation cannot apply to the breed differences in size of fowls. If mitotic rate alone were the controlling factor, the size differences should be perceptible before the 10th day as well as after it. An endocrine control was suggested by Byerly.

[2] For mice, as Kopec (1932) has shown, differences of birth weight are compensated for by subsequent speed of growth, and so equalised out.

thyroid epithelium to make "colloid" vesicles, and "colloid," is a strong argument against this view. Three possibilities exist for an explanted tissue: (1) it may merely grow, (2) it may grow and differentiate histologically, (3) it may grow and differentiate both histologically and morphologically. The otocyst is a perfect case of the second type of action, for all the tissues of the normal finished product are formed, but no spatial arrangement whatever (Fell, 1929). Embryonic limb bones show the third type, for a high degree of morphological or even anatomical differentiation is realised in their explants (cf. Fell, 1932).

The independence of histogenetic and organogenetic differentiation has recently been much emphasised by Ranzi (1929, 1931 a, 1931 b). In the course of his work on the experimental embryology of cephalopods he has repeatedly observed that the formation of the different sorts of cells characteristic of an organ may proceed quite normally, although the characteristic morphological form of the organ has been suppressed or modified experimentally. Morphological development was profoundly affected if a *Sepia* embryo was removed from its yolk and allowed to go on developing, but histological development proceeded normally.

It is clear, then, that histogenesis can occur without organogenesis, but the converse is probably also true. For in the early development of amphibians, when the neural plate is being formed, there is comparatively little histological elaboration accompanying the laying down of morphological structure. Other than by yolk content, there is little to distinguish the cells in the various regions of the embryo.

III. DISENGAGEMENT OF GROWTH, DIFFERENTIATION, AND METABOLISM.

(1) *Metabolism without growth in bacteria and yeasts.* The consideration of bacterial phenomena does not come strictly within the field of this disquisition, but it is hard to exclude a reference to them since they throw some light on the essentials of the problem. In bacteria differentiation does not come into play, and the relations between growth and metabolism can be studied without that additional complicating factor. Under natural conditions growth and metabolism are not separated in bacterial life, for if a colony finds sufficient nutrient materials to permit of existence, it will automatically enter a period of cleavage. But there are certain cases in which it has been shown that metabolism can occur in the absence of growth, so that cell division is quite absent and the cells are simply maintaining themselves as far as possible in the *status quo*.

The first of these cases is that of "resting bacteria." The term "resting bacteria" was introduced by Quastel and Whetham (1924) to mean bacteria which were respiring but not growing. The organisms were grown in tryptic broth, separated by centrifuging, washed in saline solution, made up to a thick emulsion with saline, and finally well aerated. Under these conditions no proliferation takes place and the properties of the enzymes present in the cells could be investigated without the complicating factor introduced by the syntheses of growth. The results of such investigations have been summarised by Quastel (1928) in a review. How far the

resting bacteria were alive, however, was questionable from the beginning, and attention was specifically devoted to this point by Cook and Stephenson (1928). The only available criterion of the life of resting bacteria was viability; would they divide when replaced in nutrient medium? The results of viability counts showed that only from one-third to one-tenth of the resting bacteria were able to divide when given natural conditions. Oxidations and other reactions produced by the resting bacteria, however, were not due to these viable cells alone, for when this viable minority was reduced to 0·02 per cent. of its original value by exposure to ultra-violet light, the rate of reaction was only slightly affected. Nevertheless, the presence of a considerable proportion of viable organisms among the resting bacteria demonstrated a disengagement of the growth and metabolism processes.

The second case is that of crowded yeast cells. Adrian Brown (1892) showed that with yeast growing in malt wort, the medium would only allow the cells to increase up to a certain limit, and that if this concentration were reached or exceeded in the initial sowing no multiplication took place although fermentation proceeded freely. This observation, an earlier example of the "resting" condition, permitted the study of fermentation apart from growth and enabled Brown to examine the effect of oxygen supply, etc., on the fermentation process alone. For an account of the subsequent work to which this led, reference may be made to the monograph of Stephenson (1930).

The third case is that of nitrogen fixation by *Azotobacter*. Iwasaki (1930) has recently shown that on a medium free from fixed nitrogen, cell division may occur without nitrogen fixation and conversely nitrogen fixation without cell division. The first of these conditions occurs when an old culture is diluted with new medium; the respiration rises, and cell multiplication goes on without nitrogen fixation, while the size of the individual cells decreases. After some time, when the population has greatly increased, cell division ceases and nitrogen fixation sets in; parallel with this, the cells increase in size. If the culture is very greatly diluted, a multiplication phase occurs as before, but it is followed by a simultaneous multiplication and nitrogen-fixation phase, before the colony passes over into the pure nitrogen-fixation phase. Owing to the dissociation here between cell division and increase in size of the cells, the dissociation between growth and characteristic chemical activity is not perfectly clear, but it is obvious that a certain measure of disengagement between metabolism and growth is normal to this organism. The state of affairs has striking resemblances to that referred to below in the discussion of growth and metabolism in explanted cells of higher animals.

(2) *Metabolism without growth and differentiation; embryonic hibernation and the diapause.* We do not have far to look for phenomena of a similar kind in the embryonic life of the higher animals. Many insect embryos, as is well known, complete a certain part of their development and then enter upon a state of hibernation or diapause which lasts for a considerable time, and at the end of which a short period of rapid development leads to hatching. The silk-worm egg is perhaps the most famous example of this mechanism, and it is very common among the Orthoptera (see Bodine, 1929). But during the diapause the respiration of the embryo does not

completely disappear; on the contrary, as Ashbel (1932) has shown, there is a small but continuous maintenance metabolism. The growth and differentiation processes have been thrown out of gear with the metabolic processes for the duration of the diapause.

It is not so generally known that something analogous to the diapause occurs in mammalian development. According to Reinhardt (1928), Prell (1927, 1930) and others, the embryos of the mole, roedeer, bear, badger, pinemarten, and stone-marten, pass a considerable proportion of their intra-uterine life in a state of sus-pended development. Although the subject has been quite ignored from the physio-logical point of view, there can be little doubt but that some metabolism is pro-ceeding also during this "diapause." In reptiles, embryonic hibernation is found in the case of the tuatara lizard, *Sphenodon* (Dendy, 1898), and the pond-tortoise, *Emys* (Boulenger, 1898). In birds it has not so far been reported, but certain English wild birds present suspicious behaviour from this point of view. It appears (Woodger, 1931) that the blackbird, for instance, lays one egg every day for a week, but although sitting has begun from the first day, all the eggs hatch out together. Such a remark-able arrangement merits further analysis.

(3) *Metabolism without growth and differentiation; survival of embryonic cells at low temperatures.* If natural embryonic hibernation is an instance of the possibility of suppressing differentiation and growth in a developing system, while retaining a maintenance metabolism, so is the experimental work which has recently been carried out on the effect of low temperatures on viability of metazoan cells. Buc-ciante (1931 *a, b, c*) has examined with great care the survival of embryonic cells at low temperatures. Taking hens' eggs from the incubator at definite times after the beginning of incubation, he allowed them to remain at standard lower temperatures and measured the time elapsing before it became no longer possible to make success-ful tissue cultures from the embryonic cells. As the following table shows, the various tissues had various times of survival:

	Time in days before complete loss of viability in tissue culture:	
	Left at 15–20°	Left at 0°
Skin	24	32
Cornea	18	28
Meninges	21	25
Amnios {Epithelium	7	17
{Muscle	5	10
Skeletal muscle	21	22
Spleen {Fibrocytes	6	12
{Leucocytes	3	5
Heart	6	10
Liver {Endothelium	6	10
{Hepatic cells	2	5
Mesonephros	6	12
Mesencephalon	6	10
Aorta	7	10
Iris ·	6	11

But more important for the present purpose is the fact that at these low tem-peratures metabolic processes were proceeding although growth and differentiation

had been totally done away with. Not much is known about the respiration of tissues at 0° C., but the work of Smith (1931) conclusively showed that it was not negligible. Amphibian muscle at 0° took up about 0·025 c.c. of oxygen per gm. per hour (wet weight).

Other workers have carried out experiments similar to those of Bucciante, but their durations of survival have not usually been as great as his, probably owing to the fact that they have placed the embryos or parts of embryos in saline solutions at the low temperatures instead of keeping them within the intact egg. Thus Verne and Odiette (1931) used Tyrode solution and found the survival was improved if glucose were added to it. Bucciante himself (1931 c) considers that the saline solution washes out from the tissue certain necessary growth factors, or "trephones." A similar difference of technique explains the long survivals of whole rat embryos reported by Simonin (1931) as opposed to the short survivals of Kodama (1931). Nicholas (1931) again, has successfully kept whole rat embryos at 24° in Ringer solution for 72 hours with no sign of differentiation or growth but no doubt considerable respiration.

(4) *Stepwise inhibition of growth, fermentation and respiration, by means of chemical substances and radiant energy.* So far, abnormal temperatures and naturally occurring phenomena alone have entered into the description of the disengagements which may occur between growth and differentiation on the one hand and metabolism on the other. But there are other agents which may be used experimentally to affect the gearing, and other investigations in which they have been discovered.

Of these the first example is undoubtedly that of Warburg (1908). Warburg studied the effect of hindering or inhibiting the cleavage of sea-urchin eggs by placing them in hypertonic sea water. Although profound effects were observed on the segmentation, the respiratory rate was very little changed (0·368 mg. O_2/hour/28 mg. egg nitrogen in the normal lot, 0·347 in the inhibited lot). In a later paper he described the classical case of phenylurethane. Under the influence of this drug, segmentation could be wholly abolished, and yet the respiratory rate remain unaltered, if the dose was well chosen. Thus, after 25 min. in ordinary sea water the astrospheres are visible, but nothing has happened in $N/2000$ phenylurethane. At 40 min. the first cleavage takes place in normal eggs, but in the phenylurethane eggs the astrospheres are only just appearing. At 90 min. the second cleavage takes place in normal eggs, but in the phenylurethane eggs only the equatorial plate stage has been reached. Yet in spite of this great retardation of cell division, the respiratory rate was in no case affected to a higher degree than 20 per cent. (*e.g.* 0·450 c.c. O_2/hour/28 mg. egg nitrogen in the normal lot, 0·438 c.c. in the phenylurethane lot). "The visible changes in the early developing egg," as Warburg said, "are not conditions of the oxygen utilisation following fertilisation. But the oxygenation is a condition of the visible changes, so that those chemical processes, the activity of which we can judge by the amount of oxygen consumed, would seem to underlie the morphological ones."

Experiments which show an identical disengagement of the fundamental processes were made in 1930 by Partachnikov (1930). Partachnikov studied the action of quinine on heart fibroblasts of the chick growing *in vitro*, and found that the

inhibitory effects on growth and on metabolism (as assessed by the disappearance of glucose from the culture medium) ran by no means parallel.

	Growth (Ebeling index)	Glucose utilisation %
Control	3·96	71
Quinine 1 in 10,000	0·33	58
Quinine 1 in 5,000	0·0	34
Quinine 1 in 2,500	0·0	0

Thus at a certain concentration of quinine the growth (cell division, increase in cell size, etc.) was inhibited by 100 per cent., the metabolism only 55 per cent. Higher concentrations of quinine abolished the glucose utilisation too.

A similar stepwise inhibition is seen in the results of Mikhailovskij (1929) and especially Hubert (1929). Mikhailovskij cultured explants of heart in much the same way as Partachnikov but subjected them to the action of radium emanation for periods ranging from 4 to 20 hours. Again the inhibitions of growth and metabolism were not parallel.

	Area of explant sq. mm.	Glucose utilisation %
Control	14·15	79
4 hours' irradiation	7·5	67
20 hours' irradiation	—	53

So in this case the inhibition of growth could be 47 per cent. and that of glucose utilisation only 15 per cent.[1]

Hubert's work on the chick embryo (1929) was particularly interesting in that he measured the inhibitions not only of growth and of respiration, but also of glycolysis, caused by irradiation with X-rays. He subjected 5th-day chick embryos while still *in ovo* to the action of accurately dosed X-radiation, and then measured glycolysis and respiration by Warburg manometer methods on the same day, and growth by leaving a population of embryos until the 12th day and then observing their fate. Table III summarises the results he obtained, and Fig. 3 gives them in graphic form. It is clear that the effect of X-radiation upon the fundamental processes was not at all the same; growth was the first to be affected, then glycolysis, and finally respiration. The strongest treatment of all (12,000r, *i.e.* Röntgen radiation units) caused only a 15 per cent. decrease of the respiration below normal, but glycolysis was reduced by 45 per cent. and growth entirely stopped.

X-radiation is therefore an agent which at a certain level will abolish growth but cause no change in the intensity of respiration or fermentation, while at another level it will exercise an inhibiting influence on these metabolic processes as well. The parallel with Warburg's early experiments is striking. "An inhibition of glycolysis,"

[1] Similar, but not such clear-cut, results are reported by Krontovski (1932) and Krontovski, Jazimirska-Krontovska and Sawitzka (1932) for the action of the halogen acetic acids on explanted embryonic cells.

said Hubert, "appears first at doses which lie far above the growth-inhibiting dose."
Again, "if a glycolysis inhibition appears after a latent period of 24 hours, inhibition
of growth and damage to the tissues appear as well; and one never has inhibition of
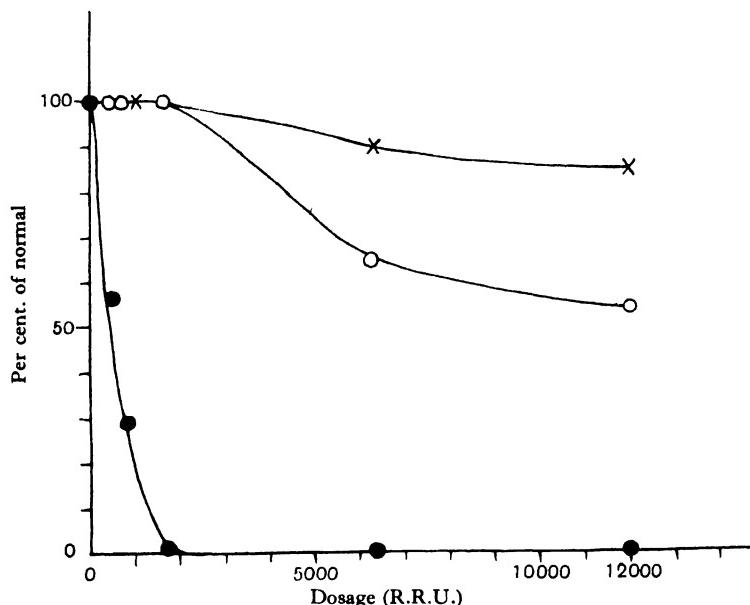
Fig. 3. Effect of X-rays on chick embryo (Hubert). × respiration, ○ glycolysis, ● growth.

Table III. *Hubert's work* (1929) *on X-ray inhibition
of the fundamental processes.*

	Normal values	Irradiation with X-rays on the 5th day *in ovo*				
		530 r	850 r	1700 r	6300 r	12000 r
Glycolysis:						
$Q_{CO_2}^{N_2}$ or NGR, measured on the 5th day, after irradiation	23·5	23·5	23·5	23·5	15·3	12·6
Respiration:						
Q_{O_2} or RR, measured on the 5th day, after irradiation	12·6	—	—	—	11·2	10·7
Growth:						
Wt. on 5th day, mg.	4	—	—	—	—	—
Wt. on 12th day, mg.	237	173 (living) 100 (dead)	111 (living) 28 (dead)	8 (all dead)	(All dead)	(All dead)

glycolysis without inhibition of growth and damage to the tissues. Inhibition of
growth is certainly not a result of inhibition of glycolysis."

So far the examples of this section have all been concerned with developing
embryonic cells, but there are two pieces of work on yeast which fit in so closely

with the argument that it is not possible to omit them. Firstly, the effect of X-rays on the fermentation, respiration and growth of yeast cells was investigated by Wels and Osann (1924). Here again a marked differential effect was obtained, growth being by far the most susceptible of the fundamental processes, and the other two, indeed, being hardly affected by the doses used. Table IV and Fig. 4 summarise the data. Just as with the chick embryo in Hubert's experiments, growth is abolished
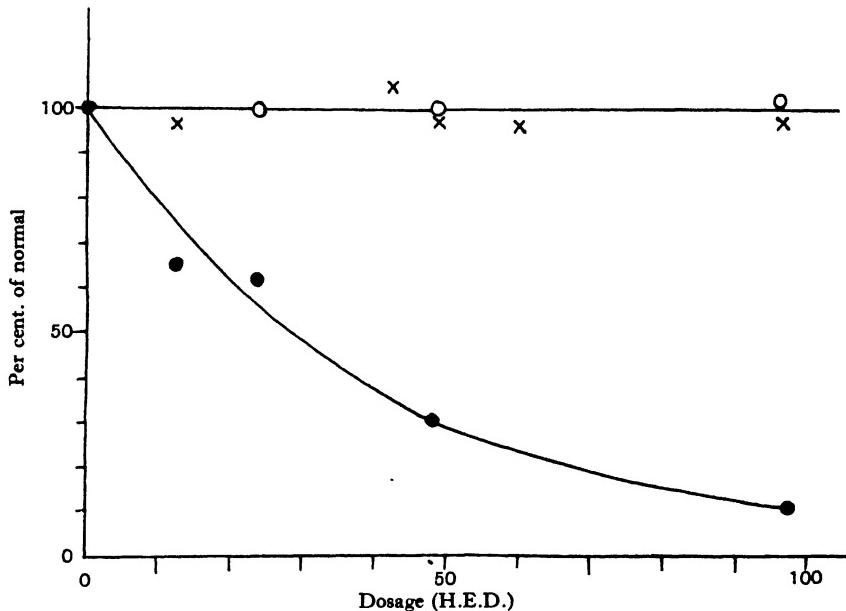
Fig. 4. Effect of X-rays on yeast (Wels and Osann). ○ respiration, × fermentation, ● growth.

Table IV. *Experiments of Wels and Osann* (1924).

X-ray dosage H.E.D. (Hauts-einheitsdosen)	Respiration % of normal	Fermentation % of normal	Growth % of normal
0	100	100	100
12	—	96	65
24	101	—	62
42	—	105	—
48	100	97	30
60	—	96	—
96	104	96	11

before any effect has become evident upon respiration or fermentation. It was interesting that cell-free fermentation showed the same immunity to X-ray damage as fermentation by the intact cells.

Similar experiments to those of Wels and Osann were carried out by Rubner (1924) as part of a general survey of the rôle of water in the living cell. By placing yeast cells in different concentrations of salt, he reduced their water content and—

when the concentration was sufficiently raised—their heat production. But only a mild degree of hypertony was required to stop growth, and as the data in Table V and Fig. 5 show, in 4 per cent. salt solution growth is almost completely suppressed while water content and heat production continue at almost their normal level. Hypertonic salt solutions, Rubner concluded quite rightly, exhibited "ganz verschiedene Relationen zwischen Stoffwechsel und Wachstum."
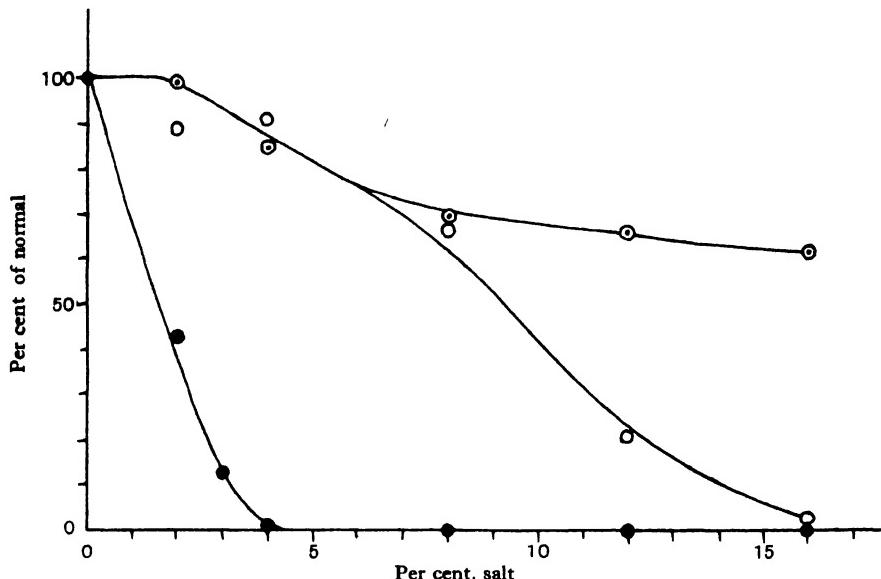
Fig. 5. Effect of hypertonic solutions on yeast (Rubner). ⊙ water content, ○ heat production, ● growth.

Table V. *Experiments of Rubner* (1924).

Concentration of salt %	Water content of yeast		Heat production		Growth index % of normal
	%	% of normal	g. cal. per hour	% of normal	
0	65·9	100	2168	100	100
2	65·1	99	1924	89	43
3	—	—	—	—	12
4	55·8	85	2004	92	0
8	46·3	70	1452	67	0
12	43·3	66	456	21	0
16	41·2	62	37	2	0

(5) *Selective engagement of growth or differentiation and characteristic chemical activity.* In the preceding paragraphs we have been considering the disengagement of growth and metabolism only in the sense of maintenance metabolism. But there are many other chemical reactions performed by the cells of an embryo, such as the storage of glycogen or the elaboration of a characteristic pigment, and it is in order

to enquire what relations exist between these special types of metabolic change and the growth processes.

On morphological grounds alone, Peter (1929) and Lauche (1925) were led to the conclusion that "a cell which is working does not divide, and a cell in mitosis is not working[1]." The subject entered a new phase when Fischer and Parker (1929) described a technique for regulating the intensity of proliferation of tissue cultures, for it was found that osteoblasts, growing under the conditions of "vie ralentie" formed bone (see also Fell, 1932), although, as Doljanski (1929) had previously shown, they would not do so when cultivated in the normal way. This indicated that growth and characteristic chemical activity might be mutually incompatible in tissue cultures, as if a primary shaft could engage with one of two adjacent gears, but not with both.

These considerations led Doljanski (1930a) to make a closer study of the relations between growth of explanted iris and melanin production. Explanted iris, if rapidly growing and repeatedly subcultured at short intervals, had been found by Kapel (1929) not to produce pigment. Doljanski now took pure cultures of iris epithelium from the chick embryo and cultivated some in the usual embryo extract, others in adult fowl plasma. The latter grew much more slowly, of course, than the former. But "while the intensely proliferating culture in embryo extract contained at the end of some time few or no pigment granules, the culture whose growth had been inhibited showed compact masses of black pigment. The granules of melanin accumulated in great quantities in the central part of the colony, and were found dispersed throughout the whole cellular membrane." Doljanski deduced an "antagonisme entre la multiplication et la fonction physiologique de la cellule."

Precisely the same state of affairs was found when liver explants were examined (Doljanski, 1930b). Ordinarily, when proliferation is rapid, the glycogen contained in the original explant soon disappears and no more is formed. But when cultures were transferred to adult fowl plasma, so that growth practically ceased or was very much slowed, glycogen was visible in abundance when the cells were treated with iodine vapour after 12–14 days. Thus hepatic cells in pure culture certainly conserve their glycogenetic power, and it only appears to be absent because growth *in vitro* is usually too rapid to permit of the characteristic chemical function of the cells. Support for this view could be found in the work of various histochemical investigators such as Jordan (1927), who concludes that the amount of glycogen in a tissue is inversely proportional to the intensity of cell division in it.

Another instance of the disengagement of development and characteristic chemical activity might be found in the work of Pozerski and Krongold (1914) on intestinal grafts. Krongold first showed (1914) that the embryonic intestine of the rat, grafted to a position under the skin of the adult animal, gives rise to a completely differentiated system, all the normally appearing kinds of cells (fusiform cells, muscle fibres, glands of Lieberkühn, mucous cells, Brünner glands, etc.) being arranged around a central lumen, the whole forming a kind of vesicle. But in spite of this

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[1] Thus, for example, cells in mitosis are not found in developing kidney tubules, if these are functional.

display of histological self-differentiation, the characteristic chemical products, saccharase, maltase, lactase, and secretin, were found by Pozerski and Krongold to be entirely missing. Enterokinase alone could be demonstrated, and even this, according to Pozerski and Krongold, was more probably of host origin, and was merely being excreted into the vesicle because of its chance presence in the host's blood stream. Histological differentiation, then, was in this case unaccompanied by the appearance of characteristic chemical activity.

On the general question of this sub-section, Fischer (1931) has assembled further relevant facts in a review. What was proved by Doljanski for explants of iris and liver finds its parallel, it seems, in explants of nerve, heart, and connective tissue, according to the results of other observers.

(6) *Cell division and differentiation without oxidative metabolism.* Cases of the disengagement of growth or differentiation from metabolism have so far been restricted to the removal of the former while the latter remains intact. But is it possible for growth and differentiation to proceed in the absence of metabolism? In the profoundest and most inclusive sense in which the word metabolism can be used, it is probably not possible, but there are not wanting suggestions in the literature of chemical embryology that the early stages of development may be facultatively anaerobic. If this were so the whole series of mechanisms within the cell which carry out its oxidations would be dissociable from the processes of growth and differentiation, which would only compulsorily engage with fermentative reactions or with reactions involving hydrogen acceptors other than oxygen.

The echinoderm egg has played its part in this, as in so many other chapters, of general physiology. Warburg, in his original experiments referred to above (p. 195), was perfectly right in claiming that segmentation of the echinoderm egg will not take place in the absence of oxygen. This compulsory aerobiosis has been found again and again by other workers (see Needham, 1931). Nevertheless, there remained the possibility that a segmenting egg might derive energy from the reduction of a reversibly reducible indicator, and might thus cleave anaerobically. The experiment was made by Reiss and Vellinger (1929). Eggs were placed in anaerobic solutions poised at different points on the rH scale by indicators, haemoglobin, and other substances, and it was found that if the potential was 200 mv. or over, *i.e.* rH 23 or above, the cleavage would proceed normally, but if it was below that point, the number of cells dividing fell off rapidly until at 150 mv. or rH 22 no more cells would divide. These results were shortly afterwards confirmed by Rapkine (1929) who used methylene blue. There had previously been studies of the effects of anaerobiosis on echinoderm eggs, but in the older experiments the cells were merely stained with methylene blue as an indicator for the disappearance of molecular oxygen, and when the dye was reduced, division ceased. In Rapkine's experiments an excess of reducible dye was present around the cells. All this work has important consequences, but we cannot yet accept the existence of cell division without molecular oxygen as definite, in view of the difficulty of removing the last traces of oxygen from a solution. Further work along these lines would be very desirable.

The special case of nematode eggs may be relevant here. Up to the present it

has been supposed that the cleavage stages are necessarily aerobic, but Dyrdovska (1931, 1932) now claims that the first cleavage can occur in pure nitrogen.

As regards the chick embryo, little is known, in view of the experimental difficulties attending the study of it in the earliest stages. But Burrows (1920) discovered a remarkable change in the respiratory properties of the chick embryo with age. Taking explants of chick embryonic heart cells, he placed them in different partial pressures of oxygen and found that their behaviour was quite different according to the stage of development of the chick from which they had been taken. Fibroblasts from 4–5-day embryos would grow and pulsate for 46 hours or so in pure nitrogen, and for 50 hours in only 7·5 per cent. of oxygen. Fibroblasts from 10–15-day embryos, however, would not grow at all in nitrogen, and only for 12 hours in 1·5 per cent. oxygen. This must mean that the younger cells possess some source of energy which the older ones have not got. Kumanomido's (1928) demonstration that the anaerobic glycolysis rate of the chick embryo declines markedly with age may here be very relevant. Burrows' work was later confirmed and extended by Wind (1926), who paid particular attention to strictness of anaerobiosis. In his conditions only the smallest amount of growth proceeded even with 4–5-day embryos, but at an oxygen concentration of $2·10^{-4}$ vol. per cent. the difference was clear-cut, for cells from 10-day embryos would not grow at all, while those from 5-day embryos grew profusely. Wind also made the interesting observation that if the cells were in each case subcultured for several weeks and then brought under anaerobic conditions, the difference between 5th and 10th day embryos had disappeared.

There is certainly no truth in the statements of the earlier workers that avian development may proceed to any length anaerobically (see Needham, 1931). But *unorganised* growth undoubtedly occurs. Byerly (1926a) allowed chick embryos to develop for definite periods up to 5 days and then, breaking off the shell covering the air space, immersed the whole egg in "water-glass" solution so that the respiratory exchange was abolished, but continued the incubation at 37° for varying periods of time. The suffocation effects included deranged body form at the posterior end, inhibition of allantois formation, the appearance of extraordinarily large blood vessels and anomalous sinuses, with fatty necrosis of the tissues. Histological examination revealed enormous quantities of blood cells, so that the haemopoeitic activity had been stimulated by suffocation. In tissues other than blood, cell division seemed to have ceased as soon as anaerobiosis became complete.

In a second paper (1926b) he continued the study of anarchistic growth. The behaviour of the tissues differed according to the age at which the heart was made to stop beating. If cardiac failure occurred after 4 or 5 days' incubation, only the liver cells and the blood cells were still proliferating at the end of a week's anaerobiosis. But if the failure took place at 72 hours' incubation or less, then, in addition to growth of liver and blood, the nervous tissue went on proliferating for nearly a week. In such embryos the mesenchyme cells seemed almost unaffected by the suffocation and continued to segment, but took on the characteristics of blood cells. The important point about the picture in these anaerobic embryos was that each

tissue became a relatively irresponsible unit, and all correlation of growth ceased with the failure of the circulation. Only unorganised cell life was possible.

The complementary research to that of Byerly was carried out in Japan some years later by Ido (1930) in ignorance of Byerly's work. On the basis of modern knowledge of cell physiology, it could have been predicted that asphyxiated embryos, if anarchistic growth (or growth without organogenesis) was proceeding, would accumulate large amounts of lactic acid. This Ido showed to be the case, in eggs treated quite similarly to Byerly's, and if cardiac failure occurred on the 7th day or later, he could exactly account for the glucose disappearing by the lactic acid formed. With earlier embryos, however, the correspondence was not so good.
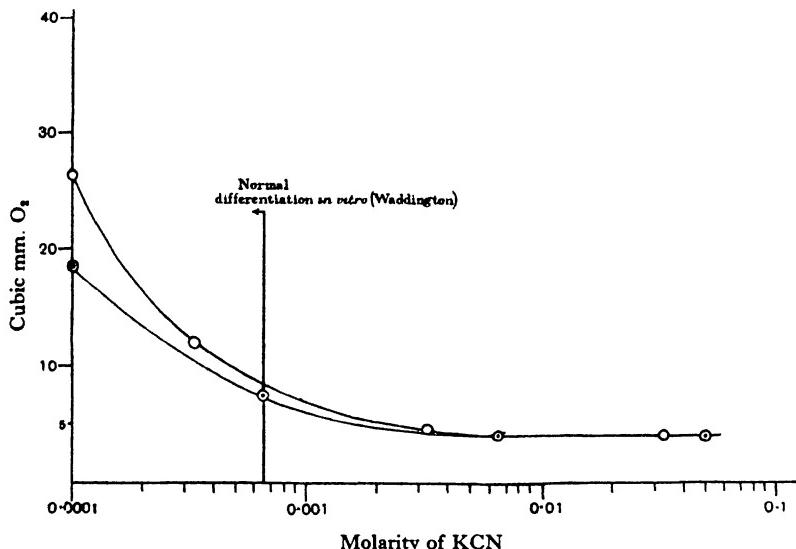
Fig. 6. Effect of cyanide on the respiration of chick blastoderm (Needham).
O 12–15 somites, ⊙ 8–12 somites. Single logarithmic grid.

There can thus be little doubt regarding the facultative anaerobiosis which chick embryo cells may manifest in assuming a fermentative life. But these cases are particularly interesting because differentiation and oxidative respiration "go into neutral," as it were, together, leaving the growth of certain special tissues side by side with glycolysis. We may hesitate, however, to generalise this two-way association. A case is known in which oxidative respiration can be severely reduced and perhaps abolished altogether, without the growth and differentiation of the embryo being affected.

When an adult tissue is treated *in vitro* with KCN, the respiration falls as the concentration used rises, until a point is reached at which further increases of concentration bring on no further inhibition. This residual respiration (Dixon and Elliott, 1929) may be some 30 per cent. of the normal respiration. Fig. 6 shows that

when chick blastoderms are treated *in vitro* with varying concentrations of cyanide, their respiration follows a descending curve quite similar to a typical curve of Dixon and Elliott's (Needham, 1932*b*). At a concentration of 0·007*M* KCN the maximum inhibition is obtained. It is probable, however, that this residual respiration is a phenomenon only shown in solutions buffered with phosphate, and that in bicarbonate or serum the inhibition of respiration would be complete at that concentration. Now in parallel experiments Waddington (1932*b*) found that the *in vitro* differentiation and growth of the blastoderms from the beginning of the primitive streak stage onwards accomplished itself quite normally[1] in KCN concentrations below 0·001*M*. Accordingly we may say that normal differentiation and growth can
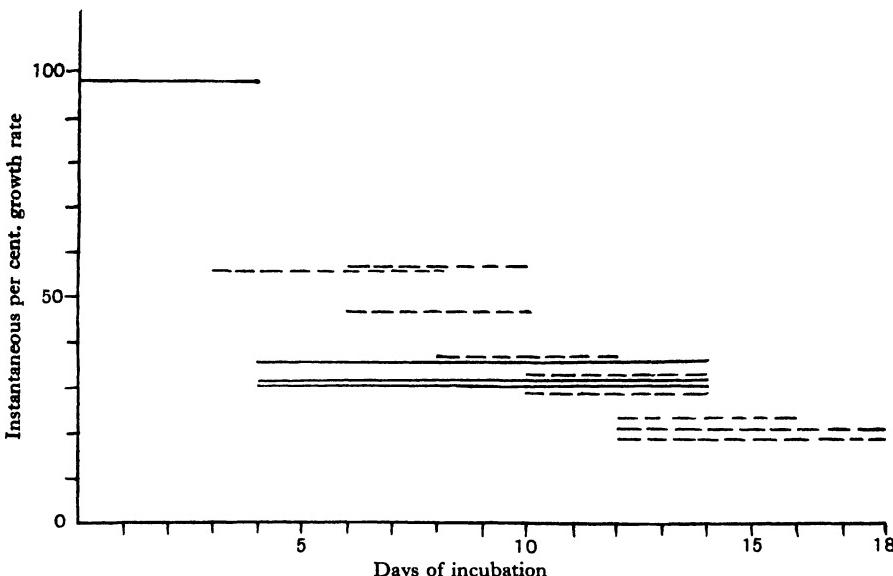
Fig. 7. Growth rates of wet weight and respiration in the chick embryo (Brody). —— CO_2 production (various workers), – – – growth (wet weight: various authors).

go on under conditions in which the normal respiratory intensity has been reduced by 70 per cent. and perhaps by 100 per cent.

(7) *Independent rhythm of the fundamental processes in the intact organism.* In addition to the actual disengagement of fundamental processes from one another, we have evidence of a certain independence of rhythm drawn from the functioning of the intact organism. Such evidence hardly comes within the present line of argument, but in so far as it shows (to continue the metaphor) that the gear wheels are of different sizes, it is relevant.

The older American workers were much inclined to assume an identity between the processes of growth and those of oxidation and nuclear synthesis (cf. Needham's account, 1931, pp. 525, 584). But the tendency received its first check when Loeb

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[1] Normally, that is, for *in vitro* development (see p. 184).

and Wasteneys (1911) published their paper "Are oxidation processes the independent variables in life phenomena?" It occurred to them to compare the temperature coefficient of embryonic development in the sea-urchin egg with the temperature coefficient of respiratory rate in the same material. The coefficients were not at all the same, a fact which harmonised, they thought, with the capacity of KCN to stop cleavage altogether while still permitting 25 per cent. of the normal oxidations. The later work on temperature coefficients and characteristics has abundantly shown that growth processes and respiration have in general an entirely different reaction to temperature change. The subject has been discussed in detail by Needham (1931, section 2·16).

Another view of the separateness of growth and metabolism in the intact organism was obtained by Brody (1927) who calculated the instantaneous percentage growth rates of wet-weight increase and of respiration. The two were quite distinct, as the values shown in Fig. 7 indicate. Early in development the respiratory mechanisms (as judged by the rate of CO_2 elimination) are increasing more rapidly than the wet weight, then, between the 4th and 7th day the position is reversed, and after that time respiration again takes the lead. "Either the CO_2-producing mechanisms develop at a constant percentage rate of the increase in body weight (during these periods) or the weight of the body and its constituents cannot be taken as an index of the growth of metabolising tissues."

IV. DEDIFFERENTIATION AND DEGROWTH.

The analogy of gearing cannot be followed far without involving the concept of reversibility. And, indeed, it appears from a survey of the known facts that a considerable degree of reversibility does exist in the cases of growth and differentiation. The question of greatest interest for the present discussion is whether the phenomenon of disengagement is shown when the processes are under reversal, just as it is when they are proceeding normally from homogeneity to heterogeneity and from smallness to largeness.

When an animal organism regresses after reaching a certain stage of development, its regression may, we find, take one of two forms; either it may consist only of a reduction in size, or it may also undergo marked morphological changes. The former of these alternatives could be called pure degrowth, the latter degrowth plus dedifferentiation. Of the first type several examples are known, but no doubt the most famous is that of the starved planarian worm, which was thoroughly worked out by Lillie (1900). When *Planaria dorotocephala* is starved, it becomes smaller and smaller until it shrinks to a size less than that at which it originally hatched from its egg. But the only morphological changes which occur are slight alterations of the proportions of the parts, and with regard to these Abeloos (1928) has shown that they retrace the alterations of the proportions of parts which accompanied normal growth. Thus measurements of the ratio pre-ocular length/total length fall upon the same curve whether the worm is growing or degrowing, and according to Abeloos and Lecamp (1929) the same statement applies to the relative size of the epithelial

cells of the gut. This reappearance of the characteristic heterogony during degrowth enhances our opinion of the reversibility of the process, but there are senses in which it is not truly reversible. Abeloos (1930) has demonstrated that the regressed planarian has not become rejuvenescent as regards growth rate; the high growth rate of normal early life is never regained, and subsequent size increase, brought about by the cessation of starvation conditions, proceeds only slowly.

There is thus no true dedifferentiation in the regression of planarians—unless the term were used to refer to the disappearance of the terminal branches of diverticulated organs, which occurs in the later stages of reduction. On the contrary, such special differentiations as the existence of subsidiary eyes persist during reduction even when the worm has sunk below the size at which it hatched. Lillie was inclined to think that reduction of cell number was alone concerned in regression, but Abeloos and Lecamp (1929) show that cell size is reduced too.

More common, perhaps, is the association of dedifferentiation with degrowth. The phenomenon has been known among ascidians for a considerable time; thus regression in colonial ascidians was discussed by Caullery (1895), and by Driesch (1902). For the present line of argument the work of Huxley (1926) on regression in the ascidian *Clavellina* is of particular interest. His illustrations of the stages in the regression are reproduced here in Fig. 8. Fig. 8*a* shows "a perfect little *Clavellina*, highly contractile, with active gill cilia and wide-open syphons, the tissues all as clear as crystal. The first sign of reduction is the permanent contraction, followed by the total closure, of the syphons, accompanied by a general shrinkage (8*b*). After a time the gill cilia stop working and the gills present somewhat the appearance of buttons. Then follows a marked shrinkage, accompanied by loss of transparency and usually by the appearance of numbers of the characteristic white pigment cells (8*c*). The opacity obliterates the gill slits in the living animal, and the shrinkage leads to the pharynx and peribranchial cavities pulling away from the ectoderm at the syphons; the one process takes place first in some cases, the other in others. More shrinkage, with consequently greater opacity, brings us to a somewhat dumpy, sausage-shaped condition (8*d*). The heart, after beating ever more slowly and more irregularly in the dense mass of loose cells which fill the body cavity, now ceases its action altogether." The organism will now have a small tail if it began with a fairly long stem stolon, but Fig. 8*e* shows a tailless example. " In the next stage, this tail, if present and not too large, is absorbed entirely, and the long oval or slightly dumbbell-shaped body of the creature shrinks into a very dense white ball, spherical, ellipsoidal, or egg-shaped. Reflected light shows it to be of a yellowish cream colour with milk-white pigment cells dotted over it; by transmitted light the pigment cells show black on a greenish ground, and here and there in the dense mass vague clearer spaces are seen, showing that organs and cavities still persist within (8*f* and *g*)." Such is the process of degrowth and dedifferentiation as described by Huxley, and it should be noted that it is not taking place in some organism low in the taxonomic scale, but in no less an animal than one of the Protochordata. Starvation and toxic agents of all kinds will produce regression, but in this particular case mere confinement in an unchanged volume of sea water was sufficient to produce it.

Now the illustrations of Fig. 8 were all drawn to the same magnification, and it is therefore very interesting to note that there is a rather sharp distinction between stages *d* and *e*. At first it would seem that degrowth occurs without dedifferentiation, for stage *d* differs from stage *a* mainly as regards its size, but later on dedifferentiation sets in, and the disappearance of organs and morphological form ensues. This may be an unwarranted interpretation of Huxley's description, but whether it is or no, the examination of regression from the point of view of a comparison between dedifferentiation rate and degrowth rate would be highly desirable. The data re-
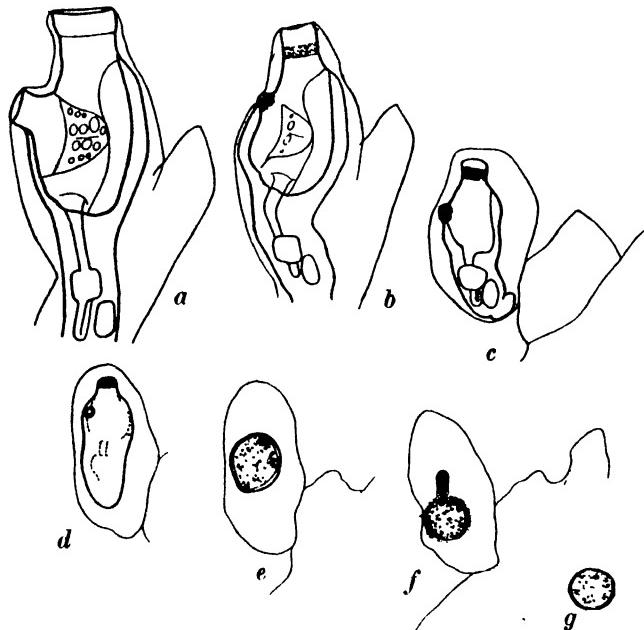
Fig. 8. Regression of the ascidian, *Clavellina* (rearranged from Huxley, 1926). (*a*) after 5 days; practically normal and still actively functional. (*b*) after 7 days; exhalant syphon closed, inhalant syphon contracting, gill slits reduced. (*c*) after 9 days; both syphons closed and withdrawn within tunic, gill slits no longer visible. (*d*) after 28 days; heart no longer beating, internal organs no l nger clearly visible. (*e*) after 37 days; a pigmented ovoid much reduced in size. (*f*) after 46 days; further shrinkage. (*g*) after 53 days; tunic omitted, slight further shrinkage.

quired for any quantitative analysis of this relationship do not seem to exist as yet in the literature.

In Huxley's opinion, the regressive changes observed in *Clavellina* cannot be supposed to represent reversions to stages passed through in embryogenesis. No ascidian tadpole makes its appearance when an ascidian dedifferentiates. The chief cell change which occurs during dedifferentiation is a regression to the cuboidal or spherical state, since such a condition requires the least amount of energy for its maintenance. These conclusions were also reached by Huxley (1921) in his work on regression in the colonial ascidian *Perophora*. But regression must be in some sense a true reversibility, since it is a passage from complexity to simplicity, from hetero-

geneity to homogeneity, from high to low organisation, from largeness to smallness, and therefore the exact opposite of embryonic development[1].

Other studies of dedifferentiation which throw light on the points discussed above are, for echinoderm plutei, Runnström (1917) and Huxley (1922), for Hydrozoa Huxley and de Beer (1923), de Beer and Huxley (1924), for Amphibia Blacher (1928). Some investigators are inclined to regard the regressed conditions as more closely allied to embryonic stages than others will admit. Thus Hadzi (1910), subjecting the medusa *Chrysaora* to starvation, obtained "gastraea-like bodies" only 80μ in diameter, composed of cells quite embryonic in appearance. The most remarkable case, however, is probably that of Davydov (1924) who studied regression in the nemertine worm, *Lineus*. By keeping portions of the worm in darkness in a constant volume of sea water under starvation conditions for long periods (up to two years) a stage was reached at which the size and shape of all the organs made the animal hardly distinguishable from an embryo in the pilidium. There was no anus, the brain was enormous, and the lateral nerve cords ran immediately under the ectoderm. In the final stage (at which Davydov had only two specimens left) a "blastula" or lump of large cells was obtained.

It is clear, then, that degrowth can exist without dedifferentiation, and that the rates of degrowth and dedifferentiation in a given case of regression by no means necessarily run parallel. Is dedifferentiation without degrowth possible? It would mean the collapse of cellular organisation and morphological form without any decrease of mass; the biological analogue of what would occur, as Robert Boyle (1744) thought[2], if the divine sustaining power were withdrawn from the universe "reducing it to a sort of Chaos or confused State of shuffled and depraved Things." It might involve both histological and morphological collapse, or it might involve morphological collapse only. Both cases occur in nature, the former in the metamorphosis of holometabolic insects, the latter in the dissociation and reunition of sponges[3].

In insect metamorphosis the situation is of course very complicated owing to the histogenetic processes which go on side by side with the processes of histolysis. "Aristotle and Harvey," writes Henneguy (1904), "thought that the larva lost all trace of organisation and in the pupa returned, so to speak, to the state of the egg. Weismann and Viallanes assumed also that the larval tissues underwent complete degeneration and that the new imaginal elements were formed at the expense of the degenerated material. More recent researches have shown that this view was erroneous. When, at the beginning of pupation, there sets in the histolysis of the larval organs destined to disappear or to be transformed, the activity of the histoblasts comes into play, and little by little the organs of the imago are built up, at the same time as those of the larva degenerate and atrophy. There is thus a general gradual transformation of the larval organs into the organs of the imago." It may,

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[1] And, moreover, the regressed ascidian will retrace its steps to normality given favourable conditions.

[2] The reference is, of course, to a posthumous edition.

[3] A most striking case of far-reaching dedifferentiation without degrowth is that of ciliates where all organelles, cirri, etc. are resorbed prior to binary fission or to regeneration after injury (cf. the interesting paper of Tavlor (1928) on *Uronychia*).

therefore, certainly be said that the larval organs suffer a process of dedifferentiation without degrowth, for their actual mass is in no way diminished, and their material elements remain behind, but in an unorganised state, until they are brought into order again by the new form of organisation. The imaginal buds, indeed, may be looked upon as similar to the blastema of a regenerating amphibian limb. Their phagocytes ingest the disorganised "pulp" to which the larval organs have been reduced by processes of dedifferentiation without degrowth (for recent descriptions, see Poyarkov (1910), Tiegs (1922) and Tai (1929). Of particular interest from the present point of view is the nature of the influence which brings about the collapse of the histological and morphological organisation of the larval tissues. Simple-minded explanations referring all to the occurrence of an acid pH appear to be inadequate for the facts (see the discussion of D. M. Needham, 1929).

Dissociation and regeneration in the sponges have been known for a very long time, but the facts are as extraordinary as those of insect metamorphosis. If a sponge is dissociated by being pressed through a sieve of gauze, the individual cells, if sown on to a glass or other surface, will come together again and in time will form a new sponge fully provided with canals, chambers, surface epithelium, skeleton and all the different varieties of cells. Recent papers of Wilson and Penney (1930) and Wilson (1932) give a clear account of the process. The normal sponge consists of the following histological elements: (*a*) epithelioid syncytial sheets lining the exterior and canal surfaces, (*b*) nucleolate mesenchyme cells, (*c*) "grey" mesenchyme cells, (*d*) rhabdiferous mesenchyme cells, (*e*) globoferous mesenchyme cells, (*f*) fibre cells, (*g*) reproductive cells, (*h*) scleroblasts and spongoblasts connected with the skeleton, (*i*) flagellated collar cells. The pressed-out material is, of course, primarily characterised by the fact that the previous morphological organisation has gone and the cells are isolated, or at any rate only bound together in very small groups. But the number of cell types in it is also limited; it contains only (*b*), (*c*) and (*i*) of the above list, though occasionally (*e*) or (*g*) may be present in negligible proportions. In all cases (*a*), (*d*), (*f*) and (*h*) stay behind with the skeleton on the gauze. Yet in spite of this absence of constituents normally essential to the life of the sponge, the reunition mass succeeds in time in producing new examples of the missing classes, presumably by the specialisation of certain of its elements. Such elements (probably the mesenchyme cells) must possess throughout normal life an undiminished embryonic potency or competence, similar to that which attaches, it is suggested, to those mesodermal cells of the Amphibia or other higher animals responsible for regenerations of lost members.

The dedifferentiation of the dissociated sponge, then, consists, firstly, in the fact that the morphological arrangement is totally destroyed by the passage through the sieve, and must be made anew, and, secondly, in the fact that the population of cells passing through is not representative of the original sponge. We cannot quite be said, therefore, to be dealing with pure morphological dedifferentiation, but the histological dedifferentiation is statistical, not individual, and thus of a very different kind from that of the histolysing cells of the insect pupa. Regeneration after dissociation does not consist primarily in the sorting out of already differentiated cells

which take up their former positions as in the normal organism. The collar cells certainly behave in this way, but the mesenchyme cells (though some of them will remain unaltered) metamorphose into the various types of cells which have remained behind.

V. INTEGRATION OR ENGAGEMENT OF THE FUNDAMENTAL PROCESSES.

The use of analogy, dangerous though it undoubtedly proved in medieval hands, is in our time perhaps almost underrated. "An analogy," says Dingle (1932), severely, "is at best worthless and at worst misleading. The analogue, not being identical with the point to be illustrated, has certain relevant and certain irrelevant elements. If the reader does not already understand the point in question, he has no means of distinguishing between them, and if he does understand it, the analogue is superfluous." However, as Dingle admits, there is something to be said for analogies. Previous experiences spring to the mind when an analogy is put forward, and the art of choosing an analogy consists in making sure that these psychological overtones will as far as possible correspond with the relevant elements of the analogue. And, above all, in certain circumstances an analogy may provide a framework or net of co-ordinates, in which a previously inchoate mass of information may advantageously be assembled.

In the course of this review I have attempted to use the analogy of mechanical gears to co-ordinate a considerable number of facts concerning the fundamental processes of ontogenesis. It has been pointed out that:

(1) Growth can occur without differentiation (explanted embryonic cells; anidian embryos).

(2) Differentiation can occur without growth (chorio-allantoic grafts; avian development *in vitro*; tissue cultures; various forms of experimental dwarfism).

(3) Nuclear division can occur without cell division (normally in arthropod eggs; experimentally in echinoderm and fish eggs).

(4) Differentiation can occur without nuclear and cell division (normally in Protozoa; experimentally in annelid and rotifer eggs).

(5) Endocrine factors can exert a differential action on differentiation and growth (amphibian metamorphosis).

(6) Dietary factors can exert a differential action on differentiation and growth (amphibian metamorphosis).

(7) The time of engagement of determinative processes with the processes of growth and differentiation can be altered (ciliary polarity in amphibian development).

(8) The relation between growth and differentiation can be affected by genetic and specific factors (large and small races of rabbits and fowls; large and small species of birds).

(9) Histogenetic differentiation can occur without organogenetic differentiation (tissue cultures of mammalian and avian cells; yolkless development of cephalopod embryos).

(10) Organogenetic differentiation can occur without histogenetic differentiation (early amphibian development).

(11) Metabolism can occur without growth (crowded yeast cells; resting bacteria; nitrogen-fixing organisms).

(12) Metabolism can occur without growth and differentiation (insect embryos in diapause; reptile and mammal embryos in hibernation; cells of mammalian and avian embryos surviving at low temperatures).

(13) Cell division, growth, respiration, fermentation, and heat production show step-wise inhibition under the influence of chemical agents and radiant energy; growth and differentiation disappearing first, then fermentation, then respiration and heat production (yeast cells; echinoderm embryos; bird embryos).

(14) Growth and characteristic chemical activity may be mutually incompatible, one or the other being engageable with the basal metabolism at one time (explants of avian and mammalian tissues; histological analysis of tissues).

(15) Cell division may occur without oxidative metabolism (echinoderm embryos in reducible dyes; asphyxiated chick embryos).

(16) Differentiation may occur without oxidative metabolism (chick blastoderms in cyanide).

(17) Growth, differentiation, and metabolism manifest a certain independence of rhythm even in the intact organism (temperature characteristics of growth and respiration; growth constants of size increase and respiration).

(18) Degrowth can occur without dedifferentiation (planarian worms).

(19) Degrowth and dedifferentiation may not run parallel in regression (colonial ascidians).

(20) Morphological dedifferentiation can occur without degrowth (dissociation and reunition of sponges).

(21) Morphological and histological dedifferentiation can occur without degrowth (metamorphosis of holometabolic insects).

Thus when the evidence is viewed as a whole, no room is left for doubt that a great deal of independence is possible between the fundamental processes of onto-genesis. In considering what we mean by dependence we may distinguish between existential dependence and dependence with regard to properties. In the first case a part or process isolated from the organism would cease to exist altogether; in the second, it would continue to exist but with modified properties. The fundamental processes of ontogenesis are evidently not existentially dependent on the integrity of the whole, but as in the case of the non-segmenting *Chaetopterus* egg, they do not take a wholly normal course when the integration has been interfered with. It is as if each fundamental process represented a layshaft which may or may not be in gear with the primary shaft, and the animal oeconomy is obviously so constituted that more than one secondary gear can be engaged with the primary shaft at one time. But in some cases, as we have seen (p. 200), there is apparently a check on this flexibility, and if growth is in gear, characteristic chemical activity cannot be. And *vice versa*. The engagement must be selective.

Now what is the primary shaft? It is probably not identifiable with one chemical reaction, but may be defined as whatever reaction the cell can carry out which will provide it with the minimum amount of energy necessary to maintain itself as a going concern in the physical world. Whatever under the worst environmental conditions suffices for basal metabolism may be thought of as the primary shaft, or rather, the automotive unit to which the primary gearshaft is attached. In auto-trophic bacteria it may be the oxidation of ferrous carbonate to ferric hydroxide, or of methane to carbon dioxide and water; in metazoan cells it may be fermentation; the esterification and desmolysis of glucose. The fact that fermentation has been found to be more susceptible to X-ray injury than respiration (see p. 197) does not detract from the force of this view, for if Hubert's experiments had been conducted

down, for before the addition of the extra amylase, lactic acid production was proceeding. Case's explanation was as follows: in the normal breakdown the amylase is in some way situated (*e.g.* on a colloidal particle) so as to be in close contact with the next enzyme in the breakdown chain, the two enzymes working simultaneously on the glycogen molecule. But in the amylase preparation added, this association with the esterifying enzyme had been destroyed. Then the added amylase combined with all the glycogen in the extract and turned it into a substance incapable of further breakdown, leaving the amylase-esterification complex with nothing to do, and so lactic acid production ceased.

What is new in these conceptions is the realisation of the part played by enzyme organisation. Case points out that the myozymase complex is extremely sensitive to rise of temperature, pronounced effects being observable by even short exposures to 37°, and suggests that this is not due to enzyme destruction but rather to the dissolution of some delicate colloidal link between one part of the complex and another. "In the living muscle itself," says D. M. Needham (1932), "a very close co-ordination of the enzyme systems is probably arranged. In muscle extract, ester may accumulate, and in the case of glycogen as substrate, there is always some accumulation of reducing carbohydrate which does not break down further. But in the intact resting or fatigued muscle there is very little reducing carbohydrate, no diphosphate, and very little monophosphate."

Oxidising enzymes, too, have been shown to possess a "morphological" character. Cook, Haldane and Mapson (1931), working with the respiratory catalysts of *B. coli*, concluded that the relation between a dehydrogenase and the oxygenase or *Atmungsferment* associated with it, is probably one of intimate juxtaposition at some intracellular surface.

These instances suffice to show the progress which is being made in the understanding of how metabolic processes engage with one another. It is probable that not until our knowledge of engagement within the metabolic realm itself has proceeded much farther shall we be able to attack the deeper problems of engagement described in this review. But there is already in existence one bold attempt to link up the chemistry of cell division with other metabolic processes, and so growth with metabolism.

This theory, which, it must be admitted, is at present largely speculative, may be associated with the name of L. Rapkine, and it can most conveniently be described in a series of sections, as follows:

(1) Agreement does not yet exist regarding the energetics of cell division (see Gray (1931) and Needham (1931)). There are workers who believe that an overgrowth of the cell leads to its division in such a way that no energy is required for the process. But there are others who believe that division cannot take place without a certain amount of energy. Rapkine (1931) suggests that this quota of energy is derived, not from oxidative metabolism, but from an intensified glycolysis brought about by a partial anaerobiosis of the cell just before division. Accompanying this there is a lowering of the oxidation-reduction potential of the cell. These events he pictures as local, confined to delicate protoplasmic regions, and therefore undetectable when gross procedures are used, such as the micro-injection of *r*H indicators.

It is clear that such a suggestion fits in remarkably well with what has been said above (notably in Section III (6)). This lowering of potential is produced, he suggests, by the liberation of sulphydryl groups (—SH) within the cell, for a linear relation exists between reduction potential and sulphydryl concentration; the more —SH the more electro-negative the potential.

(2) What is known concerning the behaviour of the sulphydryl group in embryonic growth and cell division? For some time past it has been known that the glutathione content of the embryo falls with age, just as does growth rate, mitotic rate, etc. (for the evidence see Needham (1931, section 12·7)). For example, the glutathione of the chick embryo falls from 400 mg. per cent. dry weight at the 5th
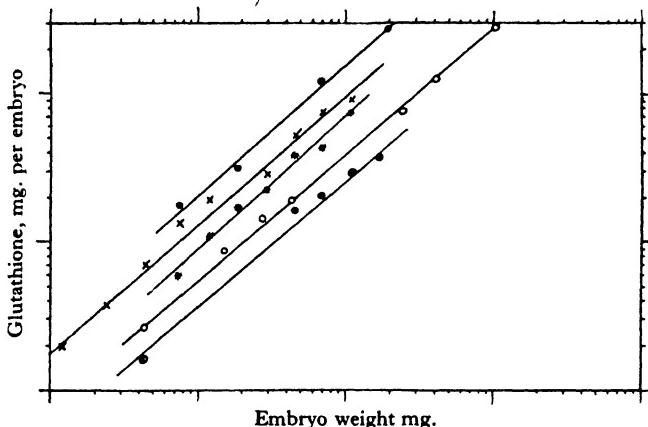
Fig. 9. Glutathione in the embryo of chick and rat (various investigators) plotted on a double logarithmic grid. N.B. The decline in —SH with age (negative heterogony) is shown by the fact that the sets of data are all inclined at an angle of less than 45° to the abscissa. It may be noted that the constant in Huxley's formula (1932) ($\log y = \log b + k \log x$; where y is the magnitude of the constituent or part, x that of the whole, and b and k constants) is very similar in all cases, even that of the rat:

		k
Chick	⊙ Murray	0·84
	• Kamiya	0·90
	× Yaoi	0·87
	● Cahn	0·88
Rat	○ Thompson and Voegtlin	0·85

day of incubation to 100 mg. per cent. dry weight at the 20th, and that of the rat embryo from 60 mg. per cent. wet weight at 0·07 gm. to 36 mg. per cent. wet weight at birth. These relationships have recently been independently confirmed by Kamiya (1930) and Tateishi (1931). Fig. 9 illustrates the fall of —SH with age in the chick and rat foetus, in the form of a double log. graph, reduced glutathione being negatively heterogonic.

As regards the echinoderm egg, Shearer (1922) stated that there was a considerable increase in reducing —SH on fertilisation, as judged by the nitro-prusside reaction. This result was accepted with some reserve at the time, but has since been confirmed rather strikingly by the histochemical work of Dulzetto (1931), who finds

a similar increase in the intensity of the nitro-prusside reaction at fertilisation. No perceptible change occurred in its intensity up to the time of the first cleavage. However, histochemical methods could not have the final word in deciding the existence of such a change, and Rapkine (1930) has now measured quantitatively the glutathione (—SH) content of echinoderm eggs during their early development. He finds that the rise at fertilisation is to a level of 40 mg. —SH per 100 gm. wet weight. It is followed by a fall to a minimum of 10 mg. per cent. at 30 min. from fertilisation, and then by a rise to a level of 45 mg. per cent. just before the first cleavage. This is fairly good proof of his thesis that segmentation is preceded by an increase of sulphydryl groups. Additional evidence is afforded by histochemical nitro-prusside reactions tried on ciliates at different stages of division by Chatton, Lwoff and Rapkine (1931) and by the work of Ephrussi (1931) on explanted cells. Ephrussi found that tissue cultures in non-nutritive media gave positive nitro-prusside reactions so long as their growth was continuing, but ceased to do so as soon as it stopped. Even if heated to 100° these exhausted cultures gave no nitro-prusside reaction, but those still in growth gave an enhanced one owing to the denaturation of the proteins (see (4) below).

(3) Quite independently, certain American workers had been led to envisage a connection of some sort between sulphur metabolism and cell division. Hammett (1928 a, b) found that the length of the roots of *Allium cepa* and *Zea mais* growing in very dilute lead nitrate solutions was inversely proportional to the concentration of lead. He also considered he had found a diminution in the number of mitoses, and no effect on cell size. If inhibited roots were transferred to solutions of substances containing the —SH group, there was found an augmentation of length as against the controls. In Hammett's view, these phenomena were to be explained by a combination of the lead with the sulphydryl group and recommencement of mitosis if additional —SH was provided. Normal growth, moreover, was found to be stimulated by the sulphydryl group, and in work with *Paramoecium* cultures, much the same state of affairs was found. In a long series of papers Hammett and his collaborators (Hammett, 1928 a, b, c, 1929 a, b, c, 1930 a, b, c, 1931; Hammett, Anderson and Justice, 1931; Hammett and Hammett, 1932; Hammett and Justice, 1928; Hammett and Reimann, 1929 a, b; Hammett and Smith, 1931; Hammett and Wallace, 1928), have extended this theory to wound healing, skin proliferation, crustacean limb regeneration, etc. In the later papers of the series, Hammett extends his views to cover the oxidation products of sulphur and claims that sulphur compounds such as sodium *p*-toluene sulphinate, di-*p*-toluene sulphoxide, diphenyl-sulphone, etc., exert an inhibiting effect upon growth and mitosis.

Hammett's views were quickly tested by a number of other workers, not with entirely satisfactory results. It is true that Voegtlin and Chalkley (1930) found both oxidised and reduced glutathione to stimulate nuclear and cell division in *Amoeba*[1].

[1] See also the later work of Chalkley (1931) on normal cell division in *Amoeba*, and of Chalkley and Voegtlin (1932) ön the effects of Cu and —SH upon it. These authors consider that the former inhibits and the latter accelerates nuclear growth. In solutions of either agent the nuclear volume of the cells at division is normal, but a greater or lesser number of cells are brought to this state of maturity and so a greater or lesser number divide.

The effect of sulphydryl compounds on the rate of development of the eggs of the snails *Physa* and *Limnaea* was studied by Gaunt (1931), however, with entirely negative results. No stimulation of development, judged by hatching time, could be found, and Gaunt threw doubt on the validity of Hammett's use of ordinary amino-acid solutions as controls rather than natural conditions. Sun, again (1930), was unable to accelerate the rate of cell division in the sea-urchin egg by treatment with H_2S. Then Morgulis and Green (1931 *a*, *b*) tried the effect of sulphydryl groups on regeneration in the polychaete worm, *Podarke*. There was no evidence of any cumulative advance in the regeneration of the experimental animals as compared with the controls. Morgulis and Green brought criticisms against Hammett's methods of calculation and expression of results, and particularly against the use of onion root tips in mitotic counts. In this they were probably quite justified, for it has recently been abundantly shown (in opposition to much, if not all, of the evidence on which the theory of mitogenetic radiation is based) by Taylor and Harvey (1931) in America, and by Moissejeva (1931 *a*, *b*, 1932) in Russia, that the rate of variation in the normal number of dividing cells in different parts of the root is enormous (see also Rossmann (1928 *a*, *b*) and von Guttenberg (1928)). Finally, there is some evidence in the papers of Baker (1929) and Coldwater (1930) that sulphydryl stimulates the growth of tissue cultures and planarian worms respectively.

On the whole, however, it cannot yet be said that the causative action of raised —SH concentration on cell division has been experimentally proved. But this does not detract from the plausibility of Rapkine's belief in a connection between sulphydryl groups and cell division, which rests on other grounds as well.

The original experiments of Hammett on lead were continued with mercury. Hoadley (1930) showed in an interesting paper that very regular inhibitory effects could be obtained by treating sea-urchin eggs with very dilute solutions of mercuric chloride. After fertilisation, he exposed them for short periods to the action of the heavy metal, and then returned them to normal sea water. As Fig. 10 (drawn from his data) shows, the relation between exposure time and time required for the first egg of the batch to cleave for the first time is practically linear. With higher concentrations of mercury in the protoplasm (longer exposure time) later development is more affected, since the curve relating exposure time to percentage of swimming embryos after 18 hours drops rather suddenly after a certain point is reached. The work was continued by Rapkine (1931) who found similarly that with mercuric chloride echinoderm development can be stopped at any given stage in 100 per cent. of the eggs, but also that it is almost completely reversible' if they are transferred to sea water containing cysteine or thioglycollic acid. This reversibility, however, varies regularly according to the time which has elapsed since fertilisation. Thus 5 min. after fertilisation 70 per cent. of the mercury-treated eggs recover if —SH is present, but 40 min. afterwards (just before the first cleavage) only 30 per cent. recover. After cleavage the percentage rises to 70 again. Moreover, Rapkine showed that similar regular variations occur in the amount of fixed —SH (the thermostable residue of Hopkins and Dixon (1922)). Finally, estimations of lactic acid,

though with an admittedly very rough colorimetric method, gave indications of regular variations corresponding to those just described.

(4) One more link in the chain of reasoning remains. Rapkine supposed (1931) that the essential phenomenon causing the increase of —SH before cell division was the increase of —SH groups attached to protein molecules, *i.e.* fixed —SH. Harris (1923) has shown that the denaturation of a protein is usually accompanied by the "unmasking" of fixed —SH groups. Thus raw egg-white is quite unreactive to nitro-prusside, but on denaturation by heat or other agents, it becomes vividly reactive. For some time the phenomenon remained of chemical rather than bio-
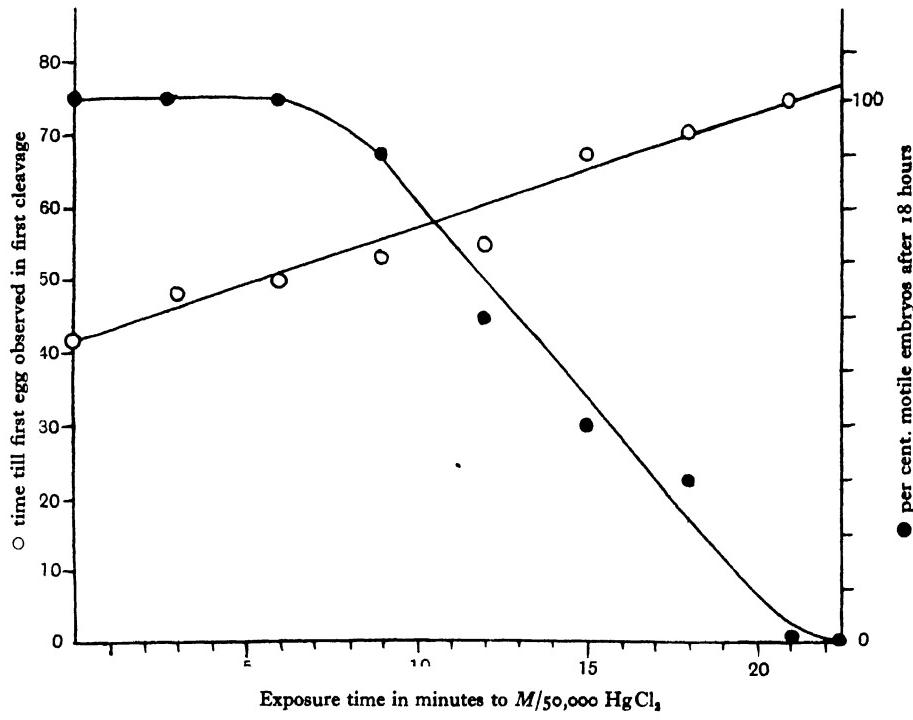
Fig. 10. Effect of Hg on *Arbacia* eggs (Hoadley).

logical significance, but with the claim of Anson and Mirsky (1931) that protein denaturation is reversible, it entered a new realm of possibilities. And when it is remembered that among the agents which can cause denaturation of proteins, certain end-products of metabolism, such as urea (Hopkins, 1931) are prominent, the significance of the fixed sulphydryl groups in Rapkine's scheme becomes apparent. It is true that the concentrations of urea required for protein denaturation are very high, but it is by no means unreasonable to suppose the existence of local accumulations in minute reaction vessels within the cell.

The original form of Rapkine's theory, then, was as follows: (*a*) protein catabolism produces urea which accumulates at certain points within the cell, (*b*) the urea

brings about a denaturation of certain proteins, (c) the —SH groups arising therefrom reduce the soluble —SS— and change the oxidation-reduction potential of parts of the cell towards the electro-negative side, (d) the resulting partial anaerobiosis intensifies glycolysis (which may be normally proceeding), and with the energy so produced, cell division is accomplished.

Now the theory as a whole is to some extent independent of its individual components, for if it should become impossible to regard the denaturation as due to urea, there are other conceivable methods (*e.g.* light, *p*H, hydration) by which it could be accomplished. Reversibility, again, is not essential, for a succession of protein molecules might be envisaged. But the point at which the theory will most need modification is the manner of réduction of the soluble —SS —.

It was previously thought that the fixed — SH of the proteins was the only agent in the cell capable of reducing the soluble —SS—, but very recently certain other systems have been found to bring this about. Hopkins and Elliott (1931) could obtain reduction of glutathione by unknown substances in surviving liver. Kodama (1932) and Tsukano (1932) find that soluble —SS— can be reduced by hexose phosphate plus an enzyme extractable from cardiac tissue and adrenal cortex. Meldrum, again (1932), reports that intact red blood corpuscles rapidly reduce glutathione in the presence of glucose and other hexoses. And Mann (1932) has recently described a water-soluble enzyme from liver which reduces glutathione in the presence of added glucose; in this case, however, other hexoses are inert. In the light of these data, the association of soluble —SH with the reversible denaturation of cell proteins becomes less convincing—perhaps not an undesirable thing in view of the fact that Anson and Mirsky's work has not yet been generally accepted. The participation of carbohydrates in glutathione reduction, on the other hand, is rather striking, and there may now be, perhaps, a more plausible connection between —SH and glycolysis than between —SH and protein catabolism.

In the end Rapkine's theory comes to this; that there is some connection between —SH and cell division on the one hand, and on the other hand there is certainly some connection between —SH and metabolic processes. It must be for future work to unravel their exact relationships. But the theory affords an instance of the kind of hypothesis we need to explain the engagement and disengagement of the fundamental processes. If we set this side by side with what we know of the synthetic activity of proteases, we have already an adumbration (though shadowy indeed) of the nature of growth.

VI. CONCLUSION.

An attempt has here been made to co-ordinate our knowledge of the dissociability of the fundamental processes in embryonic development. Co-ordination has been performed by means of an analogy, the analogy of mechanical gearing, and the fundamental processes have been envisaged as so many secondary gears engaging or disengaging, as it were, with the primary shaft of basal metabolism. Whatever the value of this analogy may be, there can be no doubt about that of the facts (sum-

marised on pp. 210 and 211) which have been brought together in this review. It might indeed have been entitled, had the author possessed sufficient Kantian audacity, "Prolegomena to any future theory of the integration of the developing organism."

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ADDENDUM.

(i) To the agents mentioned in Section III (4) which produce a stepwise inhibition of growth and metabolism, radium may be added (Krontovski, A. A. and Lebensohn, E. G. (1932), *Archiv. f. exp. Zellforsch.* 13, 407).

(ii) The argument of Section III (7) should be amplified by the important fact (emphasised by Dürken, B. (1932), *Experimental Analysis of Development*, tr. Newth, H. G. and A. M.) that determination is independent of cell-division. The two processes may have a great variety of time-relations, ranging from those found in extreme mosaic eggs to those in extreme regulative eggs, and passing through many intermediate types.

EXCHANGES OF WATER IN THE FROG

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(With four Text-figures.)

CONTENTS.

I. INTRODUCTION.

WATER is the liquid in which a huge number of living organisms exist. Among aquatic organisms the exchanges of water have been most extensively studied in the common frog; hence it is natural to present the information for this species as indicating the types of processes concerned in transfers and equilibria of water. Anatomically two groups of exchanges are present: between body and environment and between one tissue and another. Physiologically the same factors and forces operate in both groups, but, as will be seen, in diverse proportions.

An aquatic organism differs in its water relations from any other kind of living being. Terrestrial animals are clothed in hide or in chitin, which limits their intake of water to the surfaces of the alimentary canal. Terrestrial plants resist water exchanges above ground, but are essentially aquatic below ground. Aquatic organisms meet different problems, depending upon whether they live in the salty ocean or in fresh water. For, marine invertebrates and plants are very nearly in osmotic equilibrium with their environments; but fresh-water animals or plants maintain concentrated bodies in a highly dilute environment. The data to be reviewed elucidate how this result is accomplished in the frog.

II. NORMAL WATER METABOLISM.

A terrestrial organism is characterised not only by structures that conserve water but also by processes that conserve water. Thus, a man spends energy in concentrating the dissolved materials of urine to about four times the total concentration of blood. In this way he saves himself the trouble of drinking and absorbing three-

fourths of the water he would otherwise need. A frog has no power of permanently concentrating the urine above the blood (Przylecki, 1922); the frog has no use for doing so as long as it stays in water or in contact with moist ground. Further, the frog does not expend the energy necessary to swallow and absorb water; it permits the osmotic pressure exerted by its body substance to attract, through its skin, all the water that is needed. A frog drinks through its whole body surface.

But it has been found that the water intake would be much faster if the skin of the frog were not present or else were not endowed with certain peculiar properties. Normally at 20° C. a *Rana pipiens* allows 31 per cent. of its body weight of water to come in and to be excreted every 24 hours (Adolph, 1927 c). Were the skin not there, or were it robbed of its peculiar properties by the simple experimental procedure of destroying the frog's brain and keeping the frog at a high temperature (Adolph, 1931 a; Jungmann and Bernhardt, 1923), then water would at first enter five times as fast, and instead of being a trickling brook the skin would be a flowing river.

All water comes into the frog through the skin. Molecular processes move water at random in all directions; water is hence going out through the skin to some extent, it is believed. The excess of entrance over exit is due to the force of osmotic pressure exerted by the solute content of the frog's tissues, this force being greater, so long as the skin is in contact with water, than all forces tending to send an excess of water in the outward direction.

Water, in net amounts, leaves the frog chiefly through the kidneys, but it is also lost by evaporation from the lungs and skin. In ordinary life the excretion by the kidneys seems to be usually regulated to keep pace with the intake of water (Adolph, 1927 c). When the frog is removed from water, formation of urine ceases (Nussbaum, 1878; Adolph, 1927 c). When extra water is injected into the frog, diuresis develops until the excess is removed (Adolph, 1927 c).

Under some circumstances the kidneys do not excrete water so fast as water comes into the body, so that the frog swells. This happens when either the hypophysis or the entire optic lobes are removed from the frog's brain (Jungmann and Bernhardt, 1923). It also happens at low temperatures, below 5° C., and is apparently an essential process of hibernation (Donaldson and Schoemaker 1900; Ott, 1924). In addition, at low temperatures the frog does not bother to empty the bladder, and, if a frog is removed from water, water may be absorbed again into the blood from the bladder and so supply any shortage (Steen, 1929).

If the kidneys of a frog are excised or otherwise thrown out of action, water keeps coming into the frog for many hours, and thus the body swells enormously (Pohle, 1920; Przylecki, 1922; Jungmann and Bernhardt, 1923). But there is evidence (Przylecki, 1922) that the frog eventually ceases to take up water and can exist for many days without kidney function. During this period presumably many of the solutes that are characteristic of the urine accumulate until they are of concentrations where large amounts leave the body through the skin. In fact, at low temperatures frogs normally lose through the skin most of whatever urea and chloride are eliminated (Przylecki, Opienska and Giedroyc, 1922).

The normal rate of turn-over of water by the frog's body is such as to replace every $2\frac{1}{4}$ days all of the water present at 20° C. At other temperatures the rate is different and is represented (Krause, 1928), except for the very small amounts lost through the lungs, by the rates of urine output represented in Fig. 1. The rate of turn-over of water is proportional to the body weight in frogs of different sizes.

Every cell of the body is bathed in the body fluids that are continually receiving and giving up water. The cells are believed to be doing the same inasmuch as their boundaries and contents are, it is believed, permeable to water at all times. In the
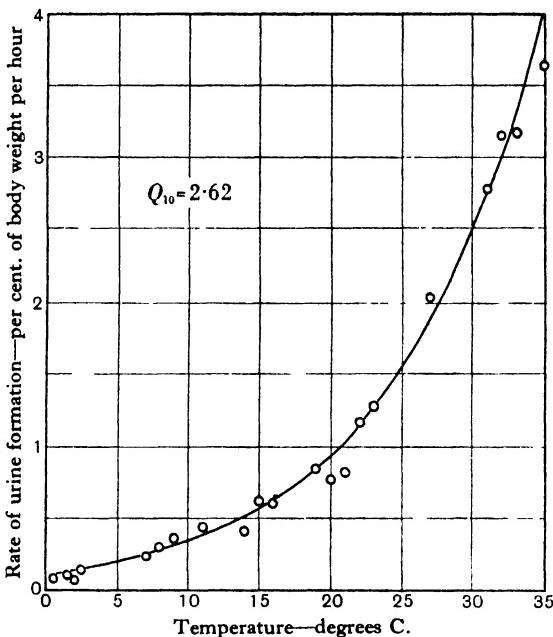
Fig. 1. Influence of temperature on the rate of water excretion through the kidneys, and hence of water turn-over. Each point is the mean of many measurements of urine caused to accumulate in the urinary bladder. These data of Krause (1928) on *Rana esculenta* are confirmed by other less complete data of Overton (1904), Przylecki *et al.* (1922), de Haan and Bakker (1924), and Adolph (1927 *c*).

midst of this stream the water content of the blood, lymph, and each tissue, as well as the total content of the whole body, is as constant hour after hour as in any mammal. Evidently all the forces concerned are working continuously to maintain a steady state with respect to water intake, output, and content.

III. RESPONSES TO SOLUTIONS.

One way of finding how the frog regulates its water content is by varying the conditions of intake. Because the frog drinks through its skin, the chief way of accomplishing this is by replacing the surrounding water by various solutions. Many solutions have been used; the most far-reaching results are to be drawn from experiments with sodium chloride solutions.

Although the frog keeps its steady state in ordinary water, and does so equally well in the purest distilled water, it gains in net content of water (Fig. 2) as soon as sodium chloride is added in concentrations less than the molecular concentration of the body fluids (Durig, 1901). Only in stronger solutions (hypertonic) is water lost. The initial rate with which water is gained can be measured at each concentration, and so the changes in the forces drawing water in through the skin are compared (Adolph, 1927 *d*, 1931 *c*) as shown in Fig. 3. Further investigation shows that the kidneys are not responsible for the increase of water content; they are excreting water just as fast or possibly slightly faster while the animal is in the hypotonic medium (Adolph, 1927 *c*) than while in tap water.
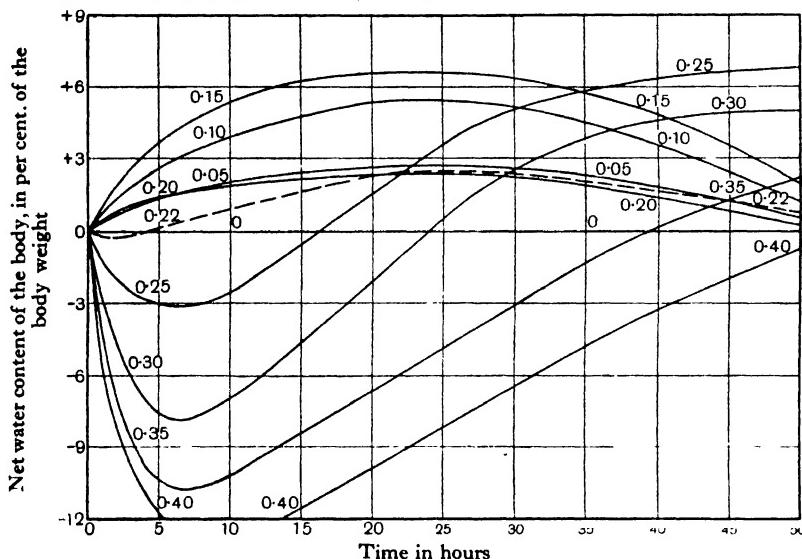
Fig. 2. Changes of relative body weight with time after transfer from tap water to various concentrations of sodium chloride. The curves are the averaged and smoothed results of many measurements on *Rana pipiens* (Adolph, 1927 *d*). The numbers indicate osmolar concentrations of the medium, unit osmolar concentration having an osmotic pressure equal to that of a molar solution of a non-dissociated and non-aggregated solute.

The best way to compare the rates of osmosis that occur in various solutions in different individuals is to refer the data obtained to the body surface (Adolph, 1931 *a*); also it has been found that the movement of water is initially proportional to the square root of the time elapsed. Thus, as much movement occurs in the second to fourth minutes inclusive as in the first minute. The body surface (S) is believed to be very nearly proportional to the two-thirds power of the body weight (W). The present data were calculated on the basis that $S = 8W^{\frac{2}{3}}$, but probably a better value for the constant is 11 (Fry, 1913; Voit, 1930) instead of 8, a correction that does not destroy the relationships to be deduced.

The rates of osmosis, when frogs are placed in various concentrations of sodium chloride, are markedly different if the frogs are first pithed (Adolph, 1931 *a*). No

longer (at higher temperatures) does water enter the animal at such moderate rates in hypotonic solutions, and in pure water the rate is enormous. The relation of initial rate of water exchange to concentration of the medium is now a straight line instead of a curve (Fig. 3). Destruction of a certain portion of the central nervous system has rendered the frog incapable of maintaining its normal water balance in fresh water. Pithing, under some conditions, increases the rate at which the kidneys excrete water (Jungmann and Bernhardt, 1923); but experiments show that it is the increased rate of entrance of water through the skin that produces the effect described. The nervous system acting upon the skin appears therefore to control the rate of entrance of water into the frog.
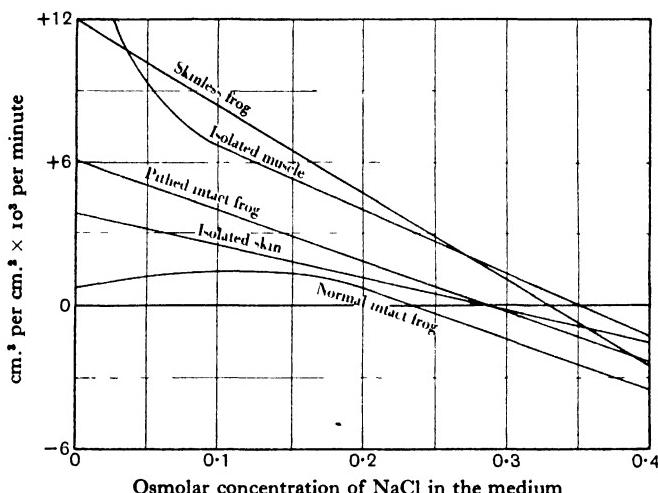
Fig. 3. Initial rates of osmosis in various concentrations of medium. Many measurements were made in six to nine diverse concentrations of sodium chloride solutions upon each of the five kinds of preparations, immediately after preparing them from normal frogs which had been in tap water (Adolph, 1931 c).

Evidence was found that the circulation of the blood was quite unnecessary in this connection (Adolph, 1931 b), though it is necessary in supplying water to the kidneys to be eliminated from the body (McClendon, 1914; McClure, 1925). But without the skin the nervous system could obviously do no regulating. Upon removing the skin (Adolph, 1931 a) and measuring the entrance of water, it was found that the entrance was again proportional to the dilution of the medium, albeit somewhat faster than in the pithed intact frog (Fig. 2). The removal of the skin has some slight effect of shortening the mean distance through which the water moves in entering the body, besides robbing the central nervous system of its effector tissue. In addition, the rate of water excretion by the kidneys is usually reduced by removing the skin. It may be mentioned that the isolated skin by itself takes up water (*i.e.* swells) in proportion to the dilution of the medium, so that the separated skin maintains its properties no more than the rest of the body does by itself.

When intact frogs are put into salt solutions, other processes than the exchange of water are modified. Salt immediately enters the body through the skin; in hypertonic solutions at enormous rates, in hypotonic solutions at very small rates (Adolph, 1927 *d*). Leaving now the initial processes that follow a change of medium, the question may be put: how do these frogs finally adjust themselves to the solutions? Eventually the passages of water and of salt are both reversed, and a new equilibrium is established at compromise values. This is especially clear when the total osmotic pressures of blood and of medium (Brunacci, 1917; Duval, 1928) are
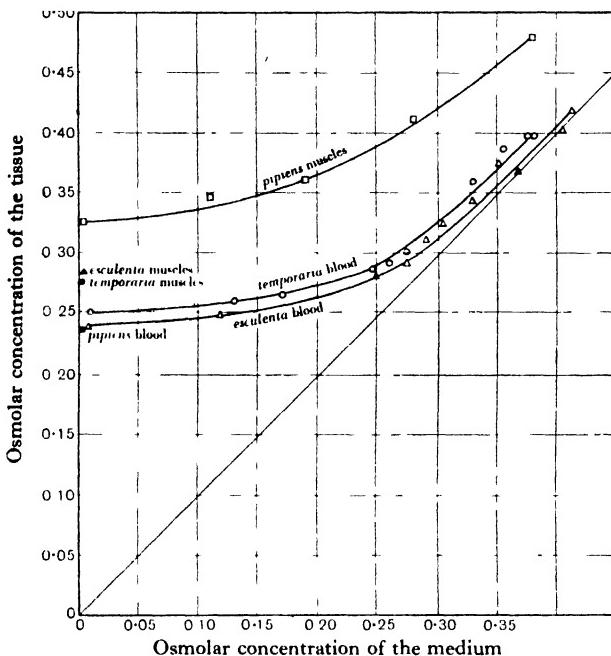
Fig. 4. Relation of concentrations (measured as freezing-points) of frog tissues to concentrations of the medium after several days have been allowed for equilibration. The graph is taken from a previous review (Adolph, 1930). The data are for *Rana esculenta* blood (Brunacci, 1917), *Rana temporaria* blood (Duval, 1928), *Rana pipiens* blood (Macallum, 1926), *Rana esculenta* muscles (Fredericq, 1902), *Rana temporaria* muscles (Backman and Sundberg, 1912 *b*), and *Rana pipiens* muscles (Adolph, 1927 *d*).

compared at equilibrium (Fig. 4). The higher osmotic pressure of the blood represents a somewhat shrunken frog, but not so shrunken as it would be if no salt had been admitted to the body. At the same time, the concentration of salt in the water of the body is never so high as it is in the medium.

This brings up the important point that the intact frog's skin is permeable to salts under some conditions (Przylecki, 1922). When isolated, the skin exchanges chloride freely and quickly with the environment, equilibrium being reached when the water present in the skin contains a concentration of chloride equal to that in the medium (Adolph, 1931 *b*). Analyses of the whole frog do not show important gains

or losses of chloride in hypotonic salt solutions (Adolph, 1927 *d*). But it is not certain that this is because the chloride cannot penetrate the skin. It is true that the frog with the skin removed loses chloride to the hypotonic medium very rapidly; the intact frog hardly at all (Adolph, 1931 *a*). But is the hypotonic medium possibly an environment that arouses no force for differential diffusion of chloride, rather than one that blocks the passage of chloride?

Experiments were done by injecting salt solutions of various concentrations under the skins of frogs, that is, into the dorsal lymph sacs. Dilute solutions were lost very rapidly, chiefly or entirely by diuresis through the kidneys (Adolph, 1927 *c*). Concentrated solutions drew water into the body before the salts were in turn excreted (Adolph, 1927 *a*). Hence both sides of the skin are sufficiently impermeable towards chlorides to enable these salts to exert some osmotic pressure effectively.

The only way to find how much of the osmotic pressure possible to salts can be effective with frog skin *in situ* as the limiting membrane is to compare the rate of water exchange caused by the salts with that caused by some substance which does not penetrate the skin under any conditions. Such a substance must be colloidal; two substances have been tried, gelatin and gum acacia. But the highest concentration of these which could be used gives an osmotic pressure less than 0·01 osmolar, which is seen on the scale of Fig. 2 to have an immeasurably small effect on the water exchanges of frogs. However, when mixed with salts, such a small concentration of gelatin has a very marked effect on the water intakes of both normal and skinless frogs. This was correlated with the influence of the gelatin upon the exchange of chloride by the frog (Adolph, 1931 *a*), an influence that in itself has not been fully elucidated. Similar effects were apparent when gum acacia-NaCl solutions or mammalian blood plasma were injected subcutaneously (Adolph, *unpublished*).

Some information as to whether sodium chloride exerts its full osmotic pressure across frog skin is supplied by comparison of its effect on water transport with that of other substances beside gelatin and gum acacia. Sucrose and glucose, to which many living tissues are not freely permeable, and other salts, particularly potassium chloride and calcium chloride (Durig, 1901; Adolph, 1925 *b*, 1927 *a*), had the same effect when in hypertonic concentrations, though they allowed less intake of water in the hypotonic range than sodium chloride allowed.

It is therefore evident that in hypotonic media there is no rapid exchange of chlorides or of other dissolved substances through the skin. There is some plausibility, consequently, in the assumption that sodium chloride exerts a considerable portion of its total osmotic pressure across the skin of the normal frog. It was found empirically (Adolph, 1931 *a*) that the amount of water exchanged (S) is proportional to the square root of the time elapsed since immersion in a salt solution ($\sqrt{t}$), to the surface area (q), and to the gradient of concentration (c) between the salt solution and the body fluids. A provisional osmotic constant might hence be calculated (Adolph, 1931 *c*). Water behaves as though it were moving towards the place where its molecules are most separated by the interposition of solute particles;

from which it might be assumed that an osmolar solution has 1 gram-molecule of water (18 gm. per litre) replaced by solute, not allowing for the fact that water is not all monohydrol. Then $c = 0.018\ (p - m)$, where m is the osmolar concentration of the particular salt solution used and p is that concentration of salt solution at which no exchange of water occurs, for p is a measure of the concentration of the body fluids as they initially exist. Finally, the amount of water exchange

$$S = 2q \times 0.018\ (p - m) \sqrt{\frac{kt}{\pi}}.$$

The relative values of the osmotic constant k remain good, whatever alternative assumptions are chosen to replace the coefficient 0.018. The values of the osmotic constant k for the pithed frog are of the same range of magnitude (Adolph, 1931 c) as for the exchange of water across walls of the capillary blood vessels of the frog under the influence of hydrostatic pressure (Landis, 1927).

A hypothesis which pictures the initial relations of intact frog skin to the passage of water through it is as follows: When the body is immersed in solutions of hyper-tonic concentrations, the skin behaves like any other tissue in allowing water to pass in response to the force of osmotic pressure at a rate comparable to its passage into a block of salt-agar. When salt is removed, the skin is instantaneously less permeable to water; so that the ideal relationship between concentration and rate of water in-take no longer holds. Instead the skin retards the passage of water; the osmotic constant progressively decreases, and is smallest in pure water. It is not enough to say that the skin is approximately semi-permeable (Overton, 1904). The environ-ment of the skin thus determines its permeability to water, an arrangement that precisely suits the exigencies of life of an aquatic organism.

The later relations of the intact frog to salt solutions are also important (Adolph, 1927 d). In hypotonic salt solutions the water initially gained begins after 24 hours to be lost. The freezing-point depression of the muscles is by this time slightly in-creased, due in part to the entrance of chloride. The new direction of regulation may be partly due to a recovery by the skin of its original resistance to the passage of water. In hypertonic salt solutions the water initially lost begins in about 6 hours to be regained (Fig. 2). The regain proceeds for many hours until the body weight is greater than its initial value. The course of events is fully accounted for by the rapid entrance of chloride into the body so that the content of chloride reaches a maximum at about 12 hours. The chloride serves to draw and to hold more water in the body. After 12 hours, salt leaves the body, allowing the water content to fall gradually to its initial value. As in an artificial osmometer, so in the frog that has lost its super-ficial semi-permeability, water is able to pass the surface more rapidly than salt, allowing initial movements of water toward a salt concentration that ultimately is not maintained. Eventually the water content of the body comes to remain at nearly its initial value while the concentration of all the body fluids is kept at the new value indicated in Fig. 4.

It has been noticed that precisely the same concentrational adjustment is found in frogs that habitually live in brackish water. But a frog has no chance of surviving

in sea water, because in such an environment the skin lets water pass out before sufficient salt has come in to prevent the enormous and rapid desiccation of the tissues.

IV. FROGS OUT OF WATER.

The frog is amphibious with respect to its mechanisms of breathing and of locomotion, but proves to be wholly aquatic with respect to water balance. This is demonstrated by putting a frog out of contact with water, whereupon it loses water by evaporation at a huge rate. If the skin is removed, evaporation proceeds at exactly the same rate as before under like atmospheric conditions (Adolph, 1932). Hence the skin contributes no protection whatever against loss of water; in contrast to all truly terrestrial animals that resist evaporation by having skins that are almost impermeable to water.

Does a frog behave toward atmospheres of various vapour tensions in the same way as toward solutions of the same vapour tensions? To answer this question requires measuring with great accuracy the losses of weight by frogs under highly constant atmospheric conditions (Adolph, 1932). Such conditions can be furnished only in still air, and in still air the rate of evaporation into a perfectly dry atmosphere is about 1 gm. of water per hour (11 mg. per sq. cm. per hour). The rate of evaporation is very closely inversely proportional to the relative humidity; the most concentrated solution in which a frog can survive is about 0·4 osmolar, which corresponds to a relative humidity of 98·6 per cent. The expected difference in rate of loss between a frog in a saturated atmosphere and one in this humidity might be of the order of 10 mg. per hour. Although the method used was capable of measuring 1 mg. per hour, the physiological variations were great enough to obscure any differences within this range. An atmosphere is the only membrane known that is perfectly semi-permeable; but under the best conditions it retards the passage of water so greatly that osmosis cannot be studied accurately with air as the medium. It may be noted that the most rapid desiccation by a current of dry air caused the frog to lose water less rapidly than the same frog gained water when put into water again.

When frogs were exposed to saturated atmospheres under rigidly uniform temperatures, it was found that evaporation still went on. Hence, under no steady conditions could a frog gain water from the atmosphere. The reason for this is one that holds for all organisms and tissues; it is that the frog is continually producing heat, thus raising its temperature above that of its surroundings, hence enabling it to evaporate water by raising the dew-point of the air in contact with its surface. It may be calculated that in 100 per cent. humidity at 20° C. one-fifth of the heat produced is lost as latent heat of evaporation (Adolph, 1932). At 94 per cent. humidity all heat produced is lost by evaporation; and at lower humidities enormously more heat is lost than is produced by metabolism, the body temperature being lowered (Hall and Root, 1930) to a point where the additional heat is gained from the environment by radiation, conduction, and convection.

A great many studies have been made of the amount of desiccation that can be endured by frogs. The limit is approximately half of the water contained in the body

(Smith and Jackson, 1931). Some investigators (Almeida, 1926) conclude that very rapid desiccation causes death at much smaller degrees of water loss, but others do not find this to be so (Hug, 1927).

The distribution of water loss during desiccation among various organs and tissues has been measured in a number of investigations (Ueki, 1924; Iizuka, 1926; Smith and Jackson, 1931; Heller, 1930 *a*). All are in agreement that in extreme desiccation lungs, brain, and eye-balls lose hardly any water; the kidneys, heart, spinal cord, stomach, intestines, and muscles lose less than the body as a whole; while the blood, skin, spleen and liver lose relatively more water than the whole body. With various amounts of desiccation the relative losses vary in characteristic ways (Ueki, 1924), the general conclusion usually drawn being that the composition of the physiologically most important tissues is longest maintained.

V. SOME OTHER INFLUENCES.

Inanition and hibernation are important in the water balance of the frog; both go together in the usual annual cycle of activities. Inanition undoubtedly changes the organic composition of tissues so that relative water contents as measured are no longer referred to the same basis as before inanition. In no case have the absolute changes of volume or weight in individual tissues been ascertained. Hibernation occurs in lowered temperatures. This reinforces the influence of inanition in increasing the percentage water contents of all tissues (Donaldson and Schoemaker, 1900; Ott, 1924). It has been found that the oedema of hibernation is characterised by low concentrations of the plasma proteins (de Haan, 1927) and by increased reabsorption of water in the tubules of the kidneys (Oliver and Shevky, 1929).

There are regular changes of water relations in frogs with age. This is to be expected in view of the many anatomical and physiological differences between embryo, tadpole, and adult. The contents of the ovary are environed by the body fluids of the adult. As soon as the eggs are laid they lose salts and become less concentrated (Backman and Sundberg, 1912 *a*); this involves little increase of volume by the entrance of water (Bialaszewicz, 1908). After fertilisation the ova swell slightly. Gradually the developing embryo gains water without gaining solids until it has hatched from the gelatinous membrane and has begun to feed (Davenport, 1897; Bialaszewicz, 1912). After a few weeks the total osmotic pressure of the larval body becomes equal to that of the adult blood (Backman and Sundberg, 1912 *a*; Bialaszewicz, 1912).

Osmosis in response to salt solutions in the environment is throughout the larval stages very different from that in the adult. In the early stages exchanges of water are, of course, very rapid because of the high ratio of surface to mass. But in all stages of the larvae water is lost upon transfer from water to any solution whatsoever. It was found that during metamorphosis a very sudden change occurred in the response to hypotonic salt solutions (Adolph, 1927 *b*). The adults always gain water in these solutions, the larvae always lose; the day after the fore-legs appear is the time of the transition, and upon this day the gills cease to function. Hence the regulation carried out by the gills is probably very different from that by the skin.

The nervous system controls the maintenance of water balance in the frog in two important ways. Its partial destruction augments the intake of water through the skin, and may, under some circumstances, stop the kidneys from excreting water. In the latter case water accumulates in the body up to enormous amounts. In the former case the kidneys may be able to augment the rate of excretion so as to keep the water content of the body normal; or they may lag behind, allowing but a mild oedema. The parts of the central nervous system whose destruction allows water to enter the body more rapidly are (Pohle, 1920; Jungmann and Bernhardt, 1923) the optic lobes, the infundibulum, and the anterior lobe of the hypophysis. If the cerebrum, spinal cord or vagus nerves are transected, no effects upon water exchange develop.

A variety of conditions appears to influence the results of brain operation, most of which have not been identified. Extirpation of the hypophysis has been some-times (Tschernikoff, 1926) found to reduce the rate of urine excretion below the normal. In frogs kept at higher temperatures destruction of the medulla oblongata stops the formation of urine (Adolph, *unpublished*). The kidneys fail to excrete water because they are asphyxiated when the breathing stops. At lower tempera-tures oxygen is adequately supplied to the blood through the skin if the breathing ceases. At the same time the rate of intake at the skin is initially augmented to the usual degree, wherefore such a pithed frog gains weight considerably faster than a frog rendered anuric by extirpating the kidneys.

Anaesthesia with chloretone inhibits the formation of urine at higher tempera-tures through depression of the breathing, while not increasing the rate of intake of water. This gives rise to an oedema. With anaesthesia by urethane or ether inter-mediate effects upon the kidneys are observed; in all cases the anaesthetic is con-tained in the medium of tap water (Adolph, *unpublished*). Anaesthesia is another means of causing the kidneys to cease their activities or to reduce their rates of urine formation while leaving the skin uninfluenced.

That there is also direct interaction between absorption of water by the skin and the excretion of water by the kidneys has been demonstrated (Richards and Schmidt, 1924; Bieter, 1930) by cutting the splanchnic nerves.

The influence of endocrine glands in the frog has been studied with respect to water balance, chiefly in the case of the hypophysis. It was found (Pohle, 1920; Jungmann and Bernhardt, 1923) that extirpation of the hypophysis caused frogs to gain water faster. This has been localised by some observers in the anterior lobe (Jungmann and Bernhardt, 1923), by others in the intermediate lobe (Tschernikoff, 1926). When extracts of mammalian hypophysis were injected (Brunn, 1921; Biasotti, 1923; Heller, 1930 b, 1930 c; Steggerda, 1931), the rate of water intake through the skin was increased, often without diuresis. Explanation of the apparent contradiction in the results of extirpation and of injection has not been completed, but many factors other than pituitary materials may be involved. An identity of mammalian and amphibian pituitary extracts has not been demonstrated with respect to water balance.

VI. ISOLATED TISSUES.

A great many investigators have attempted to solve a variety of physiological problems by studying the rate of swelling of freshly *isolated* frog muscle. It has been ascertained that total concentration, various cations, colloids, anaesthetics, fatigue, asphyxia, and temperature all modify the rates of uptake of water. Some of these results are discussed by Höber (1924). From data on these rates, attempts have been made to identify the forces concerned in water uptake. It appears that passive tension of the tissue is negligible in water uptake, in the isolated state. Osmotic pressure is highly important; but the muscle cell membranes are partially permeable to some electrolytes and so the common salts do not exert their full osmotic pressures. That forces of colloidal hydration exist has been concluded by many investigators, but equally denied by others using the same indirect methods of measuring them. There is always some uncertainty as to how much of the swelling that is measured in an immersed muscle is due to intracellular uptake of water.

The rate of water uptake in muscle has been found (Adolph, 1931 *c*) to be proportional to the surface, to the osmotic pressure of the medium over most ranges of concentration, and to the square root of the time after immersion. These facts allow an osmotic constant to be calculated, the value of which represents the velocity with which water spreads through tissue by its own molecular movements. It turns out that the constant is larger than for the intact frog.

Materials that are undoubtedly non-penetrating, such as gelatin (Adolph, 1931 *c*), exert very large influences upon water intake. This may be because excised muscles are always permeable to some of the ions of a salt solution, whereas they are never permeable to large molecules.

A few other tissues, notably stomach and kidney (Höber, 1924), have been studied in the isolated condition. So far as the data go, essentially the same qualitative relations to solutions in the uptake of water have been found as in muscle; but the osmotic constants for these tissues have not been measured.

Skin has been studied in the isolated condition in the hope that it would exhibit the peculiarities that make it efficient while on the body. Wertheimer (1925) reported that exposure to certain solutions on the outer surface only or the inner surface only made a large difference in rate of uptake of water into the skin. In most concentrations of sodium chloride at 27° C. this does not appear to be true (Adolph, 1931 *b*). Yet when the initial passage of fluid *through* the isolated skin is measured, orientation makes a difference in the presence of many solutions (Adolph, 1925 *a*). There is no doubt that skin maintains its forces of orientation for some hours after isolation, but apparently these are mere vestiges of what it had before isolation, and the forces gradually run down. The mere separation of the skin from the central nervous system might be expected to rob the skin of its resistance to the entrance of water from the outer surface.

VII. TISSUE INTERCHANGES.

Beginnings have been made in the study of fluid exchanges within the intact body. The more crude of these involve the sampling of various tissues, especially blood, muscle, and liver, after exposure of the frog to rapid water exchanges of some sort. Dehydration produces enormous changes in water content of all tissues, as was mentioned above. Changes are produced by immersion in hypertonic salt solutions (Heller, 1930 b), by injections of Ringer's solution (Conklin, 1930 a), by injections of oxytocin and pitressin (Heller, 1930 b, 1930 c; Steggerda, 1931), by fatigue (Ranke, 1865; Back, Cogan and Towers, 1915), by denervations of particular muscles (Hoffmann and Wertheimer, 1927; Heller, 1930 a, 1930 b).

The measurement of tissue volumes *in situ* has been attempted, chiefly by plethysmographs (Ellis, 1886), but the results are complicated by shifts of vascular volume.

The volume of circulating blood plasma has been measured by a dye method (de Haan, 1927; Conklin, 1930 b), but so far experimental changes in it have not been studied. Relative blood volumes have been inferred from rapid changes in the concentration of corpuscles (Isayama, 1924 a; Ito, 1926). The spleen (Bonnet, 1929) and the liver (Pellegrini, 1930) are probably concerned in the maintenance of the normal volume and constitution of the blood. After removal of the liver (Pellegrini, 1930) the concentration of plasma proteins diminishes rapidly, probably due to loss of blood, while oedema develops and body weight is gained. Without the liver the plasma proteins apparently cannot be regenerated.

More direct evidence of fluid exchanges is furnished by the experiments of Landis (1927). The rate at which fluid leaves a single blood capillary is ascertained by blocking the outflow of the capillary and finding how fast the corpuscles in the vessel march toward the site of occlusion. Simultaneously the pressure of the blood in the capillary is measured. The correlation between these two quantities is extraordinarily good, and is significantly modified by depriving the tissue of oxygen, or treating it with alcohol (Landis, 1928). At a certain low pressure the direction of transudation is reversed, and this pressure is roughly equivalent to the osmotic pressure exerted by the proteins of the frog's plasma (Krogh, 1922). It has been found (Freund, 1922; Churchill *et al.* 1927; Conklin, 1930 c), however, that proteins leak out of the blood into the tissue spaces, and appear in the lymph, at least under the experimental conditions used. Probably therefore not quite all of the osmotic pressure of these substances is effective in holding water in the blood. There is room for doubt as to whether the mean capillary blood pressure and the effective colloid osmotic pressure of the blood plasma are the only factors in the exchange of fluid between blood and extravascular spaces.

The composition of tissue fluid is believed to approximate that of the lymph which can be collected from any of the subcutaneous lymph sacs and which is regularly pumped through the four lymph hearts back into the blood stream (Churchill *et al.* 1927; Conklin, 1930 a).

The rate with which lymph is formed has been measured (Isayama, 1924 b; Ito, 1926; Conklin, 1930 a) by curarising or cauterising the lymph hearts, or by

draining the lymph sacs with cannulae. An amount of lymph equal to the volume of the whole frog's body, is found to circulate at least once a day.

The exchange of fluid across the walls of blood capillaries has been studied by perfusion with artificial fluids. Various substances have been identified as controlling the rate of transudation of fluid, particularly potassium (Gunzburg, 1918), colloids (Ellinger and Heymann, 1921; Atzler and Lehmann, 1921), and pituitrin (Drinker, 1927). The oxygen supply of the tissues is deficient in most such experiments.

The essential site of fluid exchange is between the tissue fluid and every living cell of the tissue. No direct data concerning this exchange are available. It is certain, however, that two factors are very important in the state of water distribution between them; first, the turgor that is developed in every cell so that it resists further swelling, and second, the osmotic pressure of the cell contents which is effective inside it in opposition to the osmotic pressure that is effective outside. The effectiveness in each case depends upon the permeabilities of the cell walls. The osmotic pressure is excessive inside the cells because of the high concentration of non-penetrating materials, chiefly proteins, and because of the unequal distribution of ions (membrane equilibrium).

VIII. SUMMARY.

The water content of a frog is as constant as the water content of a terrestrial animal of the same size which has access to drink. This is true in spite of the high rate at which water is continually exchanged. Constancy of water content, therefore, does not depend upon isolation.

Whereas a sac of isotonic salt solution would absorb water from the fresh-water medium at an enormous rate, the frog's high rate of absorption is only a fraction of this. The skin under the influence of the nervous system is responsible for this resistance to osmosis, since either removal of the skin or pithing the brain abolishes the resistance to the free entrance of water. Whereas normally the frog transferred to hypotonic salt solutions increases its intake of water, the operated frog absorbs water in inverse proportion to the concentration of the medium. Pituitrin administration also allows water to enter the frog more rapidly.

The kidneys excrete water as fast as it is presented to them, being capable of considerable diuresis. Under some conditions, as soon as the frog is pithed or the skin is removed urine formation ceases. Studies of isolated skin have failed to indicate the mechanism by which water is allowed to pass the skin at different rates under diverse conditions.

When out of water the frog always loses water by evaporation, even in a saturated atmosphere.

Within the body water is redistributed among the various organs under many conditions. Desiccation by evaporation, hydration and dehydration by immersion in salt solutions, injection of salt solutions, and administration of pituitrin and other endocrine substances, have been so studied. Isolated tissues behave in many respects like whole frogs when exposed to various solutions and reagents.

The conditions of transudation between blood and tissue fluid indicate that hydrostatic pressure and colloid osmotic pressure are factors. The exchanges between tissue fluid and other tissue cells have not been measured.

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PHYLLOTAXIS IN THE DICOTYLEDON FROM THE STANDPOINT OF DEVELOPMENTAL ANATOMY

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(With Eleven Text-figures.)

CONTENTS.

I. INTRODUCTION.

THE phenomena of phyllotaxis are clear evidence that the emergence and stabilisation of new lateral primordia at the growing point of a shoot take place in an ordered manner in obedience to those natural laws which govern the distribution of the increasing mass at the growing apex. In recent papers (Griffiths and Malins, 1930 and Priestley, 1929) evidence has been presented for considering each leaf primordium as the upper part of a unit of growth, the lower portion of which is the longitudinal sector of the axis which surrounds the differentiating leaf-trace and extends downwards as far as the insertion on the axis of the leaf of the growth-unit vertically below. From this standpoint, the problem of ordered succession of leaf primordia on the growing apex, is a problem of the mutual adjustment of a series of such successive growth-units to one another, and it is from this standpoint that the phenomena of phyllotaxis are now re-examined. So far the growth-units of the shoot have only been identified for the dicotyledon; in the monocotyledon the units are very differently organised, so that the treatment of these two groups of plants must be separate from the outset. The present article deals only with the dicotyledon.

II. THE EMERGENCE OF THE FOLIAR PRIMORDIUM.

Before considering the various types of phyllotaxis, a very general statement is necessary of the growth processes, to the activities of which are due the origin and subsequent development of the leaf primordia and their associated "units of shoot growth." The shoot apex consists of a dome-shaped structure of varying degrees of convexity, which is built up of a few layers of meristematic cells overlying a core of vacuolating, dividing cells (Priestley, 1928).

In the meristem the cells are growing and dividing rapidly, but being plastic structures, the cells deform as they undergo mutual adjustments and new division walls occur in various planes. In the core of vacuolating, dividing cells, the individual cells are no longer so plastic and, apart from some transverse expansion due to vacuolation, they extend mainly in the direction of the long axis of the shoot and continue to divide repeatedly by transverse walls. The resultant of these two growth processes is that the meristem, whilst spreading a little down the flanks of the apex, still tends to increase in mass in excess of the accommodation afforded by the transverse expansion of the cells of the core and can only be accommodated by an increase of surface through the formation of superficial folds. The planes of division in the meristematic layers are naturally influenced by the form changes in the plastic mass and it is general to find that, in the dermatogen or outermost layer covering all the folds, the cells, as they grow, increase in surface and always divide by anticlinal walls. The next layer immediately beneath the dermatogen, the periblem of Hanstein, behaves like the dermatogen over the actual apex, but on the flanks the distribution of the plastic cell contents leads, in places, to increased depth of the cells, followed by periclinal divisions and an increase in the number of cells at right angles to the surface. At such a position a new fold or leaf primordium emerges, in which dermatogen and subjacent meristematic layers take part, so that Schwarz (1927) has suggested the name "phyllogen" for these layers, which are also equivalent to the "tunica" of other writers (Schmidt, 1924).

In any species the manner of emergence of a new primordium remains very uniform but there seems no reason to regard such regularity as incapable of achievement by such a growth mechanism. The mechanism must be found ultimately in the protoplasmic organisation which is expressing itself in cell growth and division.

In phyllotaxis the actual locus of a new emergence becomes very important. Unfortunately all that can be said is that every meristematic apex is apparently asymmetric and growing more rapidly at one point than at any other. This point becomes the centre of a new fold. On the inner and outer surface of the fold the cells vacuolate whilst in its centre the cells remain meristematic and are compressed and elongated as they lie between the vacuolating tissues. These elongated meristematic cells, the procambial cells, probably exercise a very great influence on further development. Future growth of this primordium and subtending axis will be closely associated with their further growth and differentiation (Priestley, 1929) and their presence is probably responsible for the fact that the leaf primordium now grows much more rapidly than the meristematic apex.

In both dicotyledon and monocotyledon, the origin of a new leaf primordium is

always located over procambial tissue in the underlying core of vacuolating cells and it is true also to say that the primordium originates over a particular point in the procambial arc and from this point spreads laterally, though the point where it first emerged can usually be recognised in the developing or adult leaf and in most cases becomes the apex of the leaf. As regards the subsequent development of the primordium after its first appearance, it is necessary to distinguish the dicotyledon and monocotyledon as distinct types. In the dicotyledon, the primordium arises on the flank of the dome-shaped apex and from thence spreads laterally, but usually it does not extend more than half way around the apex and consequently the formation of one primordium does not preclude the possibility of the origin of a second at approximately the same level on the opposite side of the apex.

In the monocotyledon, the apex, which is normally dome-shaped, may at certain stages of the development of a new leaf primordium, appear almost flat. The new leaf, as in the dicotyledon, originates at one point, but then extends rapidly in length and also laterally and usually almost completely surrounds the apex at a comparatively early stage. As the primordium extends so far around the apex, the additional mass at that level is all accommodated in the single primordium.

This difference seems fundamental in the organisation of the two types and appears in the embryo, being illustrated in the paired cotyledons of the dicotyledons and the single cotyledon of the monocotyledons. In both types, the primordium grows most vigorously and is most developed above its original point of origin and consequently the next primordium to appear does so as nearly as possible on the opposite side of the apex, this being true, whether the two appear almost simultaneously as in many dicotyledons or, of necessity, one above the other as in the monocotyledons.

For both dicotyledon and monocotyledon we may, therefore, anticipate the truth of Hofmeister's generalisation (Hofmeister, 1868) as to the origin of new lateral organs upon an axis. These may be expected to appear in succession upon the opposite flanks of the growing point, because each growing fold is a competing growth centre. The next centre of vigorous growth must be established as far as possible from the one preceding it. It is worth emphasis from this standpoint that these changes are determined by the sway of the balance of growth in tissue systems which modifies rates of cell growth and distribution of cell mass and thus determines the direction of new cell walls, so that, as Sachs (1875) originally emphasised, the mutual relations of the whole system of the shoot apex determine the details of cell growth and divisions.

III. PHYLLOTAXIS IN THE DICOTYLEDON.

The usual method for describing the leaf arrangement in a plant is to express it in terms of the fraction of the circumference of the axis between one leaf and the one immediately succeeding. Thus the simplest types of phyllotaxis are 1/2 and 1/3, and other more complex types are expressed by the fractions of the Fibonacci series 2/5, 3/8, 5/13, etc., all of which have the common feature of being intermediate between 1/2 and 1/3.

Our consideration of the conditions of growth at the shoot apex has led us to expect that each successive leaf primordium should arise as far as possible from the one preceding it, and, on further examination, this fact is found to be one of the most fundamental in helping to explain the occurrence of these particular fractions. Obviously 1/2 is the maximum distance at which one primordium can be placed from its predecessor and the question arises as to why there should be any departure from this simple system, in which two leaves appear in succession on exactly opposite sides of the apex. The answer is clear, for such a system obviously becomes impossible if more than two primordia are growing at the apex simultaneously. When a number of leaf primordia are being maintained in growth activity over the meristematic apex, it will still be true that each new primordium will arise as
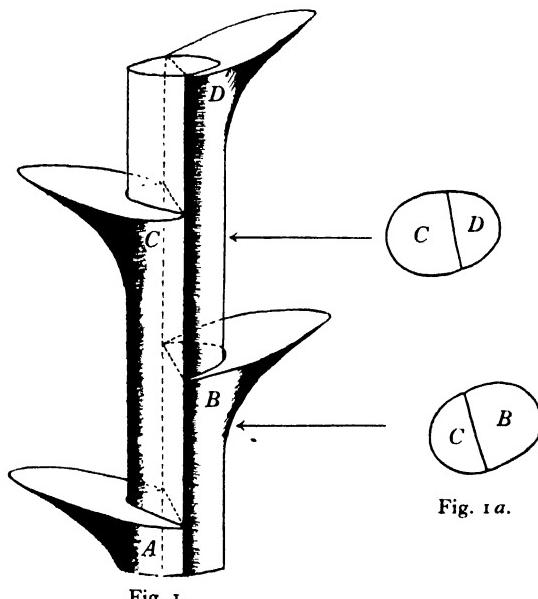
Fig. 1.

Fig. 1 a.

Fig. 1. Diagram of the arrangement of the units of shoot growth in alternate or 1/2 phyllotaxis.
Fig. 1a. Shows the contacts made by the units in transverse section.

far away from the previous primordium as possible, but it will no longer be possible for any two to be exactly opposite to one another, for the position of each will be determined, not only by the competition with the one preceding it, but also with others which spring into existence whilst it is still growing. This is more especially so since each primordium is associated in its further growth with a subtending portion of the axis, which is growing around a continuation of the same procambial strand, which will subsequently differentiate into the vascular leaf-trace common to leaf and stem.

In the case of 1/2 or alternate phyllotaxis, the arrangement of the units of shoot growth in relation to one another is simple. Thus, considering the growth-unit C in Fig. 1, it is seen to be in contact in its lower half with its predecessor, the growth-

unit *B*, in its upper portion with the younger growth-unit *D*; thus its tissues merge, in the axis, with those of the two other units, the growth of which was proceeding for part of the same period as its own. In other words, when *C* started to form, *B* was already growing; whilst *C* was still growing, *D* commenced to grow; but *C* did not originate until *A*, the unit vertically below it, had ceased to take any share in the growth activities of the apex. In 1/3 phyllotaxis, three primordia are growing simultaneously at the apex and, as a result, three growth-units are also being laid down simultaneously. As may be seen from Fig. 2, in this case, any one growth-unit such as *D* will be in contact with four other growth-units *B*, *C*, *E* and *F*. These units are so arranged that *E*, when it starts growth next to *D*, replaces the older
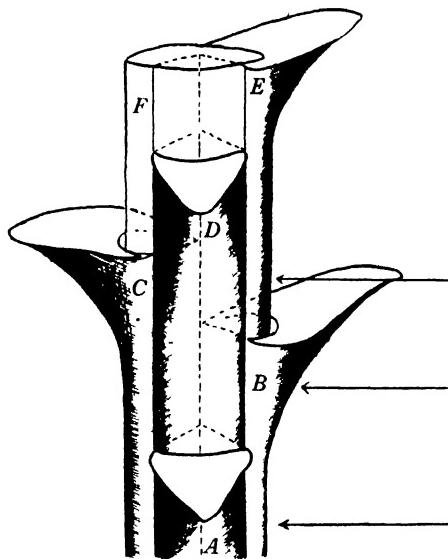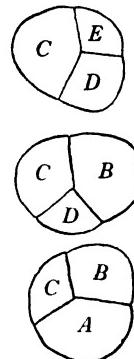
Fig. 2. Fig. 2*a*.

Fig. 2. The arrangement of the units of shoot growth in 1/3 phyllotaxis.

Fig. 2*a*. Shows the contacts made by the unit *D* at two levels.

unit *B*, which lies vertically below it and similarly *F* replaces *C*. In Fig. 2*a* the lateral contacts made by such growth-units are shown in cross section, where it is obvious that the shoot axis is organised on a sectorial basis, with a new sector inserted for each successive growth-unit as it replaces an older one which ceases to exist at that level. In this type of phyllotaxis in the dicotyledon, each new leaf primordium at the shoot apex arises only 1/3 of the circumference from the one immediately preceding it, which is not very far from the point of view of competing growth centres, each of which initiates a new growth-unit. This is probably why, in the dicotyledons, 1/3 phyllotaxis is rarely encountered except as a transient stage in the development of a more complex type. From this standpoint, the mono-cotyledon evidently has an entirely different type of shoot organisation, for, in this

group 1/3 phyllotaxis is common and is evidently a stable growth system. The number of primordia growing simultaneously will obviously be a function of the size of the apex and it is not uncommon, in the dicotyledon seedling, to find the first few leaves alternately arranged although the phyllotaxis characteristic of the adult plant is of a more complex type. As more primordia are appearing and growing at the enlarged meristematic apex, their position will be determined by the two facts, (a) that each succeeding primordium is competing with all others growing at the same time and consequently cannot arise at less than a certain minimum distance (apparently 1/3 of the circumference) from its immediate predecessor; (b) that in the subsequent development of the primordium into a unit of shoot growth, the period of longitudinal extension of any one unit will be overlapped in part by that of every other unit which was growing at the apex at the same time, i.e. extension growth of units of different ages will be proceeding at the same horizon. Thus in the higher types of phyllotaxis, when more than three units are growing simultaneously, successive units do not follow one another in a simple, regular succession, for, did they do so, two adjacent primordia would have to arise too close to one another (less than 1/3 of the circumference), and similarly in the arrangement of the sectorial units in cross section of the axis, consecutive units in order of appearance do not make actual lateral contact with one another. This explains why a phyllotaxis system does not exist with 1 as a numerator over a higher number than 3 as denominator, and why, instead, 2/5 is a possible and in fact one of the most commonly occurring fractions.

In all the more complicated phyllotaxis systems in the dicotyledons, any single growth-unit will be seen to be in contact on its flanks with four other growth-units, two older and two younger, and situated in the vertical direction between the older growth-unit which it replaced and the younger unit which only started growth as its own ceased. It has no further contact with other growth-units growing at the apex at the same time except in so far as it might be said to make contacts where the sectorial units meet in the centre of the axis.

As increasing numbers of growth-units appear and grow simultaneously at the apex, the part of the circumference occupied by any one primordium, or the sector of the axis occupied by any one growth-unit, becomes a proportionately smaller fraction of the total cross sectional area. This does not necessarily mean that the individual units are smaller: if the type of phyllotaxis in the same individual tends to get more complex as growth proceeds, the units themselves probably remain of about the same dimensions but the apex and cross sectional areas of the primary axis increase in size, so that the growth-unit merely occupies a smaller proportion of it. Nor does it seem to follow that the individual units should increase in length, though, as the phyllotaxis increases in complexity, each unit will extend through a greater number of internodes, for the nodes merely represent the points where successive primordia arise at the apex and consequently where later their free foliar parts join the parts which are coalescent in the axis.

Before passing to a more detailed consideration of the various phyllotaxis systems, one further aspect needs attention. When a plant is well embarked upon its

relatively stable, vegetative phase, it is naturally to be anticipated that the successive primordia emerging at the apex should differ little from one another in vigour of growth, that each should be an approximately equal drain upon the food supplies, and consequently an approximately equal interval of time should elapse between the inception of each. In fact this time interval should be uniform for the plant so long as it continues to grow vigorously and the leaves are arranged in the manner characteristic of the plant. Schüepp, following Askenasy, has termed this time interval the *plastochrone*, and emphasises its significance as the basal time unit, in terms of which successive morphological changes at the shoot growing point are best measured (Schüepp, 1916).

If leaf primordia of equal vigour appear at equal time intervals, their effect upon each other should be equivalent, and therefore, successive primordia should lie at equal angles of divergence from one another. Let us consider then a system in which four leaf primordia are growing simultaneously instead of three. We have already seen that they cannot arise in succession as in a 1/4 phyllotaxis, because two successive primordia are then too close to one another at the apex. If they appear, as it were, north, south, east and west, then successive ones will be separated from one another sometimes by an angular divergence of 1/2, sometimes by one of 1/4, which does not represent a stable system if the time interval remains uniform, and in fact we find no such system in which the leaves of four series appear separately at equidistant nodes. We do, however, find a system with pairs of opposite leaves at each node with successive pairs at right angles to one another so that the leaves of a pair are separated from one another by a divergence of 1/2, but the last leaf of one pair and the first of the next are only separated by a divergence of 1/4. In this decussate system, then, angular divergences between successive leaves obviously vary, whilst on the other hand, this is compensated in some way by some modification of the time intervals between the successive leaves. This is clearly a derived system and will be discussed to best advantage after an examination of the more straightforward spiral systems such as the 2/5 system, when five primordia are growing simultaneously at the apex.

(1) *The 2/5 phyllotaxis system and the Fibonacci series.*

As we pass from the condition where two or three primordia are growing at the apex, *i.e.* 1/2 or 1/3 phyllotaxis, to one in which five are all growing simultaneously, we find that the numerator of the fraction denoting the system has risen to two. This means that if one traces the series of five primordia around the axis, passing from one to the next in the order of their appearance, the tracing will have made the circuit of the circumference twice. This is obviously necessary considering the natural tendency of the primordia to arise on the opposite side of the apex from the one preceding in order of appearance. Consequently, as seen in cross section in Fig. 3 *a*, primordium 7 is flanked by 5 and 9 and similarly every other of the five sectors will be seen to be flanked on either side by a growth-unit, which is at least two plastochrones removed from it. This is a stable system since the angular divergence between successive primordia is always the same and it will also be a possible system, provided that primordia which are two plastochrones apart, such as 7 and 5, continue

to grow satisfactorily when separated from one another by not more than 1/5 of the circumference. From our previous consideration it has been seen that 1/3 of the circumference is apparently the limiting degree of proximity for the origin of successive primordia, so that 1 and 2 could not continue to grow only 1/5 apart; that 1 and 3 can do so shows that, though both are still growing at the apex, because they are farther removed in time they are also sufficiently far apart from one another in space, taking into consideration not merely angular divergence, but also distance outwards and downwards over the surface of the cone.
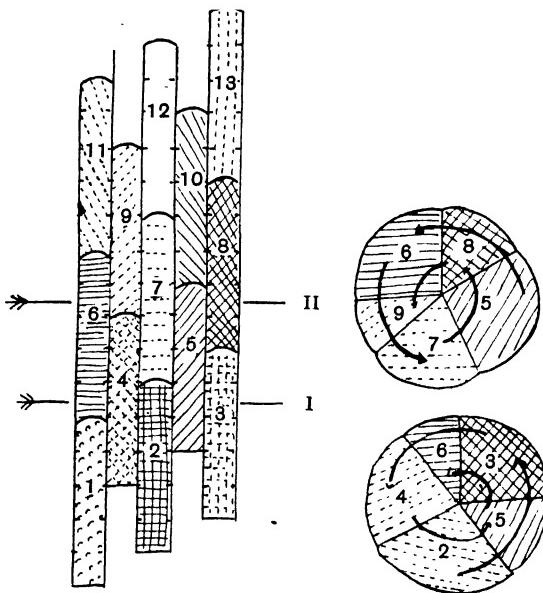
Fig. 3.   Fig. 3a.

Fig. 3. The arrangement of the units of shoot growth in 2/5 phyllotaxis, depicted as though the stem were cut open longitudinally and flattened into one plane.

Fig. 3a. The transverse sections corresponding to levels I and II, to show the lateral contacts made by unit 5.

With this conception of phyllotaxis, it is necessary to have some method of depicting diagrammatically the growth units and their relative position to one another. The solid construction is not represented sufficiently either by a study of the successive points at the apex where the primordia arise or by the relation of the primordia to one another as seen in transverse section. It is helpful to imagine the stem opened along the longitudinal plane on one radius and the cylinder of fused growth-units spread out in one plane (Fig. 3). It is then seen that such a unit as 5 is flanked in its lower portion by 2 and 3, units which were growing before it started, and in its upper portion by 7 and 8, units which have started growth since. Consequently no single transverse view can depict all the contacts made by the unit 5; if the section is shown for position I, then 5 is seen in contact with 2 and 3,

but if it is shown for position II, it is in contact with 7 and 8 (Fig. 3*a*). If the cylinder is depicted flattened out in the manner suggested, all the contacts made by a growth-unit may be shown and in the 2/5 system it will be seen that no unit is in contact with any other that is less than two plastochrones removed from it and of the four contacts made with any particular unit, two are two plastochrones removed and the other two, three plastochrones removed.

The Fibonacci series. The examination of the 2/5 system of phyllotaxis has shown us that in the ordered succession of primordia, at least 1/3 of the circumference must separate successive primordia and at least 1/5 primordia which are two plastochrones apart. If no closer distance is possible, then we have the key to the fact that the next fractional angular divergence found as phyllotaxis mounts in complexity, falls in the Fibonacci series. The mathematical properties of this series, 1/2, 1/3, 2/5, 3/8, 5/13, etc., are very numerous and are doubtless related to its occurrence in phyllotaxis. The numerator and denominator of successive fractions are obtained by adding together the numerators and denominators respectively of the two preceding fractions; the denominator of any fraction will be found as the numerator of the fraction next but one in the series, whilst the whole series may be represented by the continuous fraction

$$\cfrac{1}{n+\cfrac{1}{1+\cfrac{1}{1+\cfrac{1}{1+1}\cdots}}}$$

This series of fractions, since it merely states the angular divergence between successive leaves, may be depicted by joining a series of points with this divergence on the periphery of concentric circles, when it represents the projection of a spiral, often termed the "genetic spiral." It follows directly that the numerator of the fraction will give the number of times that the spiral passes round the circumference before a leaf is encountered which is immediately above or below the starting point and similarly the denominator must give the number of leaves in such a complete coil of the spiral between one leaf and the next vertically above or below. The latter figure must also represent the number of orthostichies or vertical rows of leaves on the axis.

Since each successive primordium must arise farther from the previous primordium than 1/3 of the circumference it follows that this fraction must always lie between 1/2 and 1/3. A fraction greater than 1/2 does not add a new fraction as it can always be read as a spiral in the opposite direction. But the Fibonacci series does not represent all the possible fractions whose values lie between 1/2 and 1/3 and the simplest way to visualise why the other fractions are usually eliminated is to write them all down and consider them in more detail. It will then be seen that between 2/5 and 3/8, there is one other possible fraction, namely 3/7. In Fig. 4, this arrangement is depicted diagrammatically and compared with 2/5 and 3/8, when it is obvious that the test of efficiency in this case is not the distance between successive primordia, but between primordia which are two plastochrones apart. In 2/5

phyllotaxis primordia 1 and 3 are 1/5 of the circumference apart, in 3/8 they are 1/4, but in 3/7 they are only 1/7 apart. The relative infrequency of 3/7 phyllotaxis may reasonably be ascribed to the fact that it brings too close together primordia which are growing simultaneously although two plastochrones apart in times of origin. (Another way of presenting this argument will be found on p. 256.)

In the series of fractions between 3/8 and 5/13, those which come under consideration are 4/9, 5/11, 5/12 and 4/11. Evidently the first three are ruled out because they bring primordia 1 and 3 too close together, *i.e.* less than 1/5 apart, whilst in the last fraction, 4/11, although 1 and 2 and 1 and 3 are sufficiently spaced from one another, apparently 1 and 4 are too near, being only 1/11 apart. Thus the essential property of the Fibonacci series is that the angular divergence represented by the

Fibonacci series—1 and 3 more than $\frac{1}{5}$ apart

1 and 4 more than $\frac{1}{8}$ apart

Fraction bringing 1 & 4 less than $\frac{1}{8}$ apart

Fractions bringing 1 and 3 less than $\frac{1}{5}$ apart
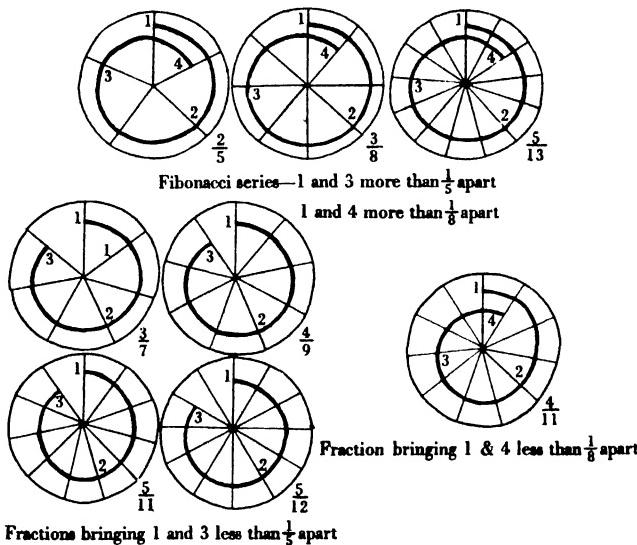
Fig. 4. For description see text.

fraction will always place primordia which are growing at the apex at the same time sufficiently far apart from one another, taking into consideration the number of plastochrones which separate them in origin. Some of these critical divergences may be read directly from the fractions, for it appears that, as a general rule, the least fraction of the circumference which may separate adjacent primordia, which are a certain number of plastochrones (numerator) apart, is indicated by the fraction thus:

Primordia 2 plastochrones apart may not be nearer than 1/5 of the circumference.

„	3	„	„	1/8	„
„	5	„	„	1/13	„
„	8	„	„	1/21	„

And any fraction which does not fulfil all these requirements will not occur at all generally in phyllotaxis.

(2) The validity of the mathematical and geometrical treatment of phyllotaxis.

This conception of the Fibonacci series regards it as determined by conditions at the apex of the growing shoot which may be formulated as a simple extension of Hofmeister's rule. Each successive primordium tends to be as nearly opposite its predecessor as possible, allowing for the fact that the primordium two before it in origin is also as nearly as possible opposite to this same predecessor and is still growing. Consideration of this system of development shows that it will lead to angular divergences between successive primordia, which will approximate to the continuous fraction

$$\cfrac{1}{2+\cfrac{1}{1+\cfrac{1}{1+1}} \cdots}$$

As Chauncey Wright expressed it "each new or higher leaf falls over the angular space between the two older ones nearest in direction, so as to subdivide it in the same ratio k, in which the first two or any successive ones divide the circumference" (Wright, 1873). Chauncey Wright regarded this as a system, as Bonnet (1754) had the spiral system in general, to distribute the adult leaves over the axis as uniformly as possible in view of their function. Church points out the inadequacy of this standpoint (Church, 1901, *loc. cit.* p. 8). We now see it as an inevitable tendency during development, determining the spacing of the growing centres as they succeed in regular order at the shoot apex. This continued fraction, the final term of the convergent series 1/2, 1/3, 2/5, 3/8, etc., is therefore "hardly devoid of biological significance" as d'Arcy Thompson (1917) puts it in his interesting chapter on phyllotaxis (*loc. cit.* pp. 635–51). Indeed one point made by him is exceedingly suggestive when the attempt is made to interpret the angular divergence between leaf primordia in terms of structures appearing in succession at a growing apex. He points out that this continued fraction bears a relation to the "*Sectio aurea*" or "golden mean"; the ratio in which a line must be divided so that the square on the larger part shall equal the rectangle enclosed by the whole and the smaller part.

The ratio in which the line must be divided may be expressed in terms of the continued fraction, which also denotes, as fractions of the periphery of the axis, the angular divergence separating successive primordia in ideal systems of spiral phyllotaxis, the wider distance approximating to

$$\cfrac{1}{1+\cfrac{1}{1+\cfrac{1}{1+1}} \cdots}$$

or $1/2\ (\sqrt{5}-1)$ and the smaller to

$$\cfrac{1}{2+\cfrac{1}{1+\cfrac{1}{1+1}} \cdots}$$

If a line AB is divided in this ratio at X and the square erected on AX and the rectangle enclosed by AB and BX, it will be seen that an asymmetric figure results (Fig. 5a). The opposite side of the square may then be divided in the same ratio at Y and the new square and rectangle erected as before, when it will be seen that the new figure (shaded in the diagram) is enclosed within the original, leaving as residue an area shown unshaded in the diagram. Obviously this process could be repeated indefinitely by successively dividing opposite sides of the resulting squares in the same ratio and constructing the figures as before. In each case a new "square-rectangle" figure is produced by removing a piece of a characteristic shape. Figures with this property of always restoring the original form if removed from or added to a given system are known as "gnomons." In our particular method of construction and considering the residual piece as the gnomon, the latter is an asymmetric figure
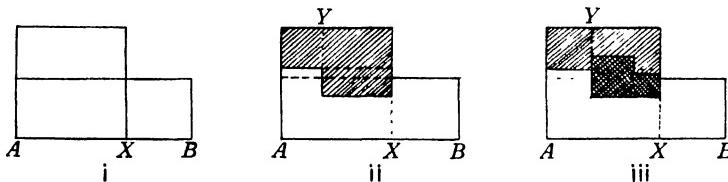
Fig. 5a.
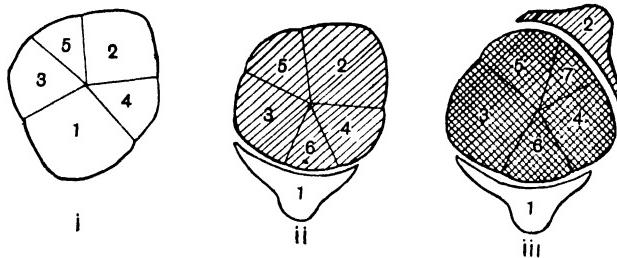
Fig. 5b.

Fig. 5. For description see text.

taken first from one side and then the other, leaving in each case another asymmetric figure, with a bias on the opposite side to that from which the gnomon has just been removed, and so the process continues with a constant sway from one side of the system to the other.

In the growth of a shoot, in which the leaves are left with an angular divergence that falls in the Fibonacci system, we are concerned with the removal of successive, separate primordia, which are asymmetric to the axis and often asymmetric in their own construction also (Hirmer, 1922). In this case after the removal of a primordium, the size of the central system is restored by the processes of growth, but the asymmetry of the system is retained and the next primordium or "gnomon" is removed from the opposite side when it reaches the same size.

It therefore seems to be more than a coincidence that the angular divergence, which expresses the ratio in which a linear dimension, the periphery of the growing

point, is shared between the successive primordia, approximates to the ratio of the "*Sectio aurea*" or "golden mean." If the emergence of the successive primordia in a 2/5 phyllotaxis system is reconstructed in plan, as seen when looking down upon the growing point (Fig. 5*b*), it will be seen that if the process is figured at the same stage in each case, the removal or addition of a figure comparable with the asymmetric "gnomon" of Fig. 5*a* would restore the original form.

It is easy to see that if attention is paid alone to the mathematical ratio which best subserves the placing of these successive, competing, sectorial growing centres around the circumference of a growing apex regarded from above, the result can be a treatment of phyllotaxis which rapidly loses touch with actuality. From the present standpoint, the ideal angle of divergence (de Candolle, 1865), to which the fractions of the Fibonacci series are converging, is of such dimensions that no two points upon the axis following within the series are ever accurately superposed. Regarded from this aspect, there is a tendency to exaggerate the difficulty of fitting any actual system of leaf insertion into a particular spiral system. So long as the leaves are well separated on the axis, leaf 6 may appear to be vertically above 1 and the phyllotaxis 2/5, but when the leaves are closer together, the eye may recognise a lack of exact correspondence between 1 and 6 and so passes beyond to leaf 9 or 14 for one vertically above 1 and so the systems 3/8, 5/13, etc., are detected. The differences in angular divergence with the fractions after 5/13 in this series are, however, less than one degree of arc so that, to quote Church (1920) "once phyllotaxis is committed to this series of fractions...the remarkable superstructure stands or falls according to the correctness of the original series, based...on orthostichies which cannot be proved to be straight and angles which cannot be measured."

That the orthostichies are often not straight and parallel with the axis was indeed recognised as early as 1837 by the brothers Bravais (1837), who introduced the term curviseriate for such systems as distinct from rectiseriate for the types with orthostichies parallel with the axis. Whilst a theoretical system of points without dimension might be spaced around an axis at this ideal angle of divergence with no successive points accurately superimposed, it is clear that leaf primordia, which make demand upon space in all dimensions and which subsequently extend, forming the sectorial growth-unit around the developing vascular strand, cannot thus form successively at the growing point without the new leaf primordium, in its turn, displacing to some extent some other primordium which now no longer actually retains a position in the meristematic apex. This primordium, left as a foliar organ behind the growing apex, need not have another primordium accurately vertically above it, but for all practical purposes, as its tissues begin to vacuolate, it is superseded in the region of the true meristem by the origin of a new fold, which will be situated more or less vertically above it.

For reasons already discussed, the relative positions of the leaf members scattered over the axis as the result of the sequence of growth processes, will usually be a spiral that can be denoted in terms of some member of the Fibonacci series, though it is not surprising that the adherence is not strict, and other fractions may occur though less frequently. A more important question, perhaps, is how complex the system can

become, not as points of insertion but as systems of growth-units, a question which obviously cannot be answered without correlation of the phyllotaxis system with anatomy. One case that may be analysed from this point of view is *Iberis amara*, for which the course of the bundles is given by Nägeli (1858) and cited by de Bary (1884). In this case the phyllotaxis in the terminal shoot is 5/13, but examination of the course of the single leaf trace bundle in the axis shows that it only remains separate through about 5–7 internodes. For example, in Nägeli's scheme (Fig. 6) it will be seen that the bundle of leaf 9, for instance, travels more or less vertically through 5 internodes when it is nearly above the trace of leaf 4, but at this point the bundle is always diverted to the left and a little lower fuses with a bundle which, higher in the axis, supplies the traces to leaves 14, 19, 24, etc. This means that in any one internode there can be detected five separate foliar strands associated with definite units of shoot growth whilst between these are sectors, which if followed up are found to be the basal parts of the next series of 5 growth-units above. Thus
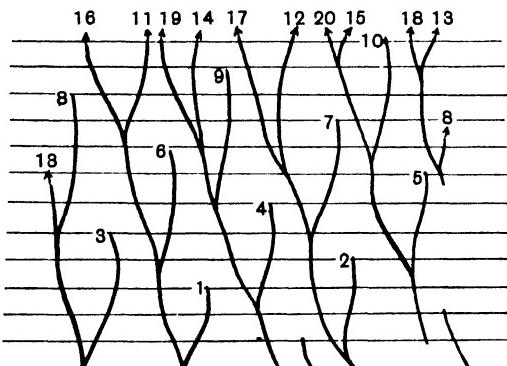

Fig. 6. Diagram illustrating the course of the leaf trace bundles
in *Iberis amara* (after Nägeli).

considering the internode below the insertion of leaf 9, development took place primarily around the strands 9, 10, 11, 12 and 13, but also to some extent around the strands supplying leaves 14–18, whilst during the same period of growth, the strand to supply primordium 19 is seen to separate from that of 14 and that to supply 20 from 15: the strand for 21 is not represented in this internode but will separate at a higher level from 16. That is to say, in this system, which externally is 5/13 and in which therefore we might expect to find evidence for the simultaneous activity of 13 growth-units at any level, in the internode analysed we have recognised the vascular strands of units 9–20 inclusive, *i.e.* 12 units; if the internode below leaf 6 is analysed in the same way, it will be seen that 13 units were growing during its differentiation, so that we may regard *Iberis* as providing evidence that in this case the identification of the 5/13 arrangement from external features is supported by the internal anatomy.

The procedure of tracing the course of the growth-units in the axis is a valuable corrective to the mental attitude resulting from the study of the Fibonacci series and

the genetic spiral as mathematical or geometrical abstractions (de Candolle, 1865; Iterson, 1907; Schoute, 1913). The higher members of the series 5/13, 8/21, 13/34, etc., may represent closer approximations to the "golden mean" and the ideal angle of divergence, but the lower members of the series are obviously more likely to be realised in the structure of the normal shoot, formed by the activity of a succession of primordia, which compete actively with one another for the available food materials and space.
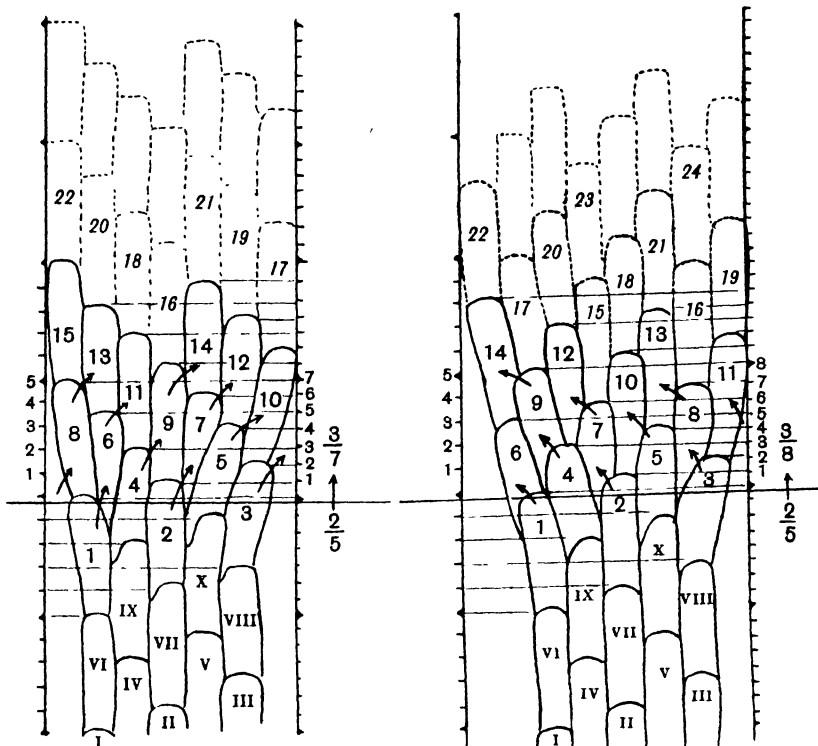

Fig. 7. Diagrams illustrating the rearrangement of the units of shoot growth when there is a transition from a 2/5 phyllotaxis to either a 3/7 or a 3/8. In the text, the lateral contacts made by the units are compared at the levels shown by the horizontal lines. The direction of displacement is indicated by the arrows.

Using the convenient mode of presentation in which the cylindrical stem is regarded as split open along the vertical line of union of two series of units, it is possible to visualise the type of change actually involved in the transition from a lower to a higher type of phyllotaxis. In Fig. 7 an attempt is made to show how an enlargement of the apex with the possibility of the simultaneous growth of more primordia might lead from a 2/5 either to a 3/7 or a 3/8 phyllotaxis. It will be seen that if the primordia continue to appear in the same relative position and if the original spiral is in the same direction in both cases, to arrive at these different systems it is necessary to make the displacement in opposite directions in the two

cases. The direction of displacement is probably indifferent so far as the spiral is concerned, but it is necessary to visualise the transverse section so as to see how the new primordia which have been introduced at any level are situated in the two systems as regards competition with their neighbours. In the type of displacement leading to 3/7 phyllotaxis, it will be seen that the following units become neighbours, 1, 6, 4, 2, 7, 5, 3, 1, and consequently units which are respectively 5, 2, 2, 5, 2, 2 and 2 plastochrones apart are separated from one another in each case by 1/7 of the circumference. On the other hand, in the derived 3/8 system, the neighbours become 6, 1, 4, 7, 2, 5, 8, 3, 6, which are respectively 5, 3, 3, 5, 3, 3, 5 and 3 plasto-chrones apart and are separated by 1/8 of the circumference. Thus in the latter system, units separated by only two plastochrones are never neighbours and the units are more uniformly distributed in space and time. We have thus visualised, in perhaps a more concrete manner, the argument already employed in a slightly more abstract form, to account for the more frequent occurrence of the 3/8 than the 3/7 phyllotaxis system (see p. 250).

One further consequence of the transition to a higher type of spiral phyllotaxis may also be considered. The change would appear to be determined by a tendency for more primordia to be maintained in growth at the apex simultaneously. We have, however, already suggested evidence for considering the leaf of a plant species as a characteristic structure in the manner of its development and also in the extent and period of its activity as a growth-unit, so that, so long as the growing apex remains of the same size, it should not be possible for a larger number of primordia to be maintained in growth activity simultaneously. The increase in number of primordia undergoing simultaneous growth must therefore mean a larger shoot apex, and also that, although the units share sectorially a larger periphery, each individual must occupy a proportionately smaller area. As the individual units thus become narrower sectors of the axis, their centres of gravity tend to move outwards, with the result that the pith becomes relatively more conspicuous and the separate identity of the growth-units harder to trace. Flot (1907) pointed out that at the growing point the pith was more conspicuous in spiral and decussate shoot systems than in alternate, and Griffiths and Malins (1930) were able to trace the limits between one unit and another across the centre of the internode in an alternate type but not in the decussate.

(3) *The genetic spiral.*

Regarding the problem of leaf insertion from the standpoint of development, attention naturally shifts from the larger number of leaves scattered around the adult axis to the primordia which are still growing at the apex and especially to the two most recent, since their growth activities will mainly determine the position of emergence of the next. In this growing region there is clearly no question of orthostichies or parastichies, since these can only be traced by the repetitive pattern formed by the insertions of the adult leaves on the cylindrical axis. Similarly the idea of the genetic spiral, which has so dominated our ideas upon phyllotaxis, has little significance when regarded from the developmental standpoint. It is clear that

if the conical apex bears upon it two growing primordia which are less than half the circumference apart, the next will of necessity appear in the wider gap between the two, and thus the position of 3 is determined by the positions of 1 and 2 and so on, and the normal result of this simple developmental system will be a succession of primordia, following one another in a spiral sequence, the direction of which, however, was determined by the position of the two original primordia and not the position of the primordia by the spiral. The spiral would be better termed a "derived" than a "genetic" spiral.

It is not surprising, therefore, to find, as the Bravais brothers (1837) point out for ·*Centaurea paniculata* and *Coniza squarrosa*, that on the branches the spiral may be right or left handed, regardless of what it is on the main axis of the same plant. They note further that the direction of the spiral on the branch is determined by the position of the first leaf on the branch, as would be anticipated from the present standpoint. If a branch system is placed so that the branch lies between the eye of the observer and the main axis, then the first leaf will be on the right or left side of the branch, if to the right the spiral will be constantly dextrorse, if to the left sinistrorse; thus, in each case, the spiral continues from the first leaf to the second by passing between the branch and the mother axis. The Bravais brothers point out also that the leaf subtending the branch, whilst contributing to the phyllotaxis system of the main axis, has also as a rule to be regarded as a member of the spiral of the branch, though the angular divergences between this leaf and the first one or two definitely on the branch are usually somewhat irregular. The point that emerges from this discussion is that the elucidation of the spiral follows when we regard it as determined by the position of the first two primordia formed in connection with the development of the branch, regarding the subtending leaf as the first.

This derivation of the spiral from the positions of the first two or three primordia formed upon the axis becomes all important when we proceed to consider whorled systems, the decussate system being treated first and at some length, as the simplest type of whorl.

(4) *The decussate system.*

Much discussion has centred round the interpretation of the decussate system. The Bravais brothers (1837) noted that in many cases the alternate whorls were not exactly superposed and that the system could consequently be described in terms of two spirals (bijugate). In certain plants, such as *Peperomia*, they noticed that the whorl was better interpreted as an approximation to each other of certain members of a genetic spiral. Around this genus controversy has since developed, Schoute finding evidence in it for derivation of the whorl from the spiral (Schoute, 1925) and Goebel on the contrary, for the spiral from the whorl (Goebel, 1913). Goebel concludes that just as 1/2 appears to be the basic system for the monocotyledons, so the decussate would seem to be for the dicotyledons.

From the standpoint of development, we have already seen that the "genetic spiral" must be regarded as a consequence and not a cause, and derived from the successive appearance of new primordia at equal intervals of time. The decussate

system, on the contrary, obviously suggests the simultaneous appearance of two primordia, the growing point going through a series of changes symmetrical about planes at right angles in successive cycles, associated in each case with the development of a pair of primordia. In this case it seems unavoidable to modify the use of the term plastochrone and to apply it to the time interval between the emergence of successive pairs of primordia, as has been done by Schmidt (1924).

It is very suggestive that decussate phyllotaxis is almost restricted to the dicotyledons (Nägeli describes one case from the monocotyledons) where the primordia occupy a sectorial fraction of the apex, thus permitting of the activity of more than one at the same horizontal level of the apex at the same time. One result of this is the prevalence of the dicotyledonary habit and another the frequent occurrence of decussate or other whorled growth systems.

It is important to bear in mind, however, that though two units may develop practically simultaneously and within the same plastochrone, they still have the
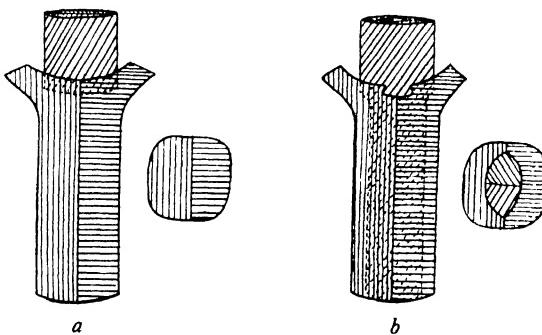

Fig. 8a. Diagram illustrating the units of shoot growth in a decussate system according to Čelakovsky.
Fig. 8b. Revised conception in which the units are considered to extend through two internodes.

same constitution, consisting of a leaf and the subtending sector of the axis. In a previous paper from this Department (Griffiths and Malins, 1930) reasons have been given for modifying the original units of shoot growth suggested for the decussate system by Čelakovský (1901) (Fig. 8a), since it has been recognised that in any internode, four units are represented by the tissues around the vascular systems of the two pairs of leaves inserted at the two nodes immediately above. In the development of this system, when a pair of primordia has been produced, the meristematic apex extends more rapidly between them so that the next pair arises in a plane at right angles. These four primordia continue to grow simultaneously at the apex for some time and eventually the lower half of the axial region of the units derived from the upper pair is more or less surrounded and fused with the upper half of the lower pair (Fig. 8b). There are many indications, when the decussate system is examined closely, that the two leaves of a pair represent quite distinct growth-units. They emerge at the apex completely separate from one another and in many cases one member of the pair is a little earlier in appearance and more vigorous in development than the other.

One of the main differences to note between this system and the spiral is that the angular divergences, instead of all being the same, are 1/2 between the individual leaves of a pair, but only 1/4 between the last leaf of one pair and the first of the next. This angular divergence is apparently sufficient because the time interval between the last of one pair and the first of the next will be longer than that between the emergence of successive single primordia of similar size upon a growing point of equal mass; the primordia presumably make the same relative demand upon the growing shoot system as when inserted singly at the nodes. There are very few observations upon the development of the same species in which comparison is
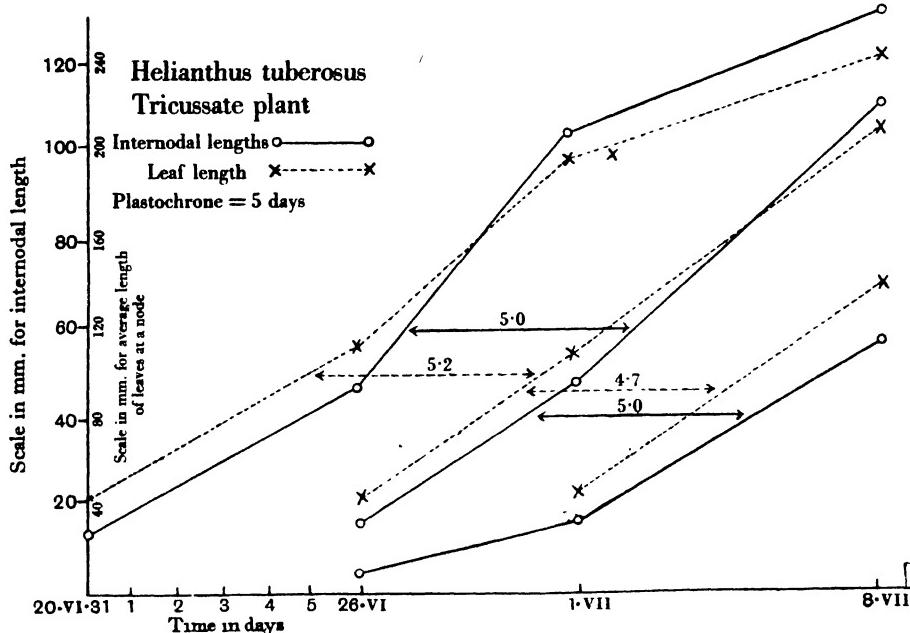
Fig. 9. Graphical representation of the growth of leaves (line entire) and internodes (line dotted) in *Helianthus tuberosus* with tricussate phyllotaxis. The plastochrone, measured by the horizontal distance between the lines for successive leaves or internodes, has an average value of 5 days in each case.

possible between spiral and decussate systems, but Schüepp (1916) has determined the plastochrone in *Helianthus annuus* with spiral phyllotaxis at 5·6 days and with decussate at 11 days, where the time interval is measured between successive pairs. This type of observation has been extended to certain other plants. In *Helianthus tuberosus* various types of phyllotaxis are shown by different plants, the majority being either decussate or tricussate, either of which systems appears to be stable, and a few spiral, in which however a tendency towards the decussate type is indicated by the occasional formation of two leaves at the same level or separated by an abnormally short internode only. The plastochrones were determined by periodic measurements of the lengths of internodes or leaves on actively growing plants. The successive measurements for the same leaf or internode, plotted against time, gave a growth

curve and where such curves for two succeeding leaves or internodes were parallel, the horizontal distance between them, parallel to the time axis, gave the plastochrone. In the case of decussate or tricussate types, the average leaf length of those inserted at the same node was taken and it was found that the plastochrones thus determined on average leaf length agreed with those determined on internodal length (Fig. 9). In this plant for a pair or a whorl of three leaves the plastochrone was consistently of the order of 5 days: evidently in the tricussate plants the apex is sufficiently large to allow of the origin of three simultaneous growth-units in the same time interval as that in which two originate in the decussate. This is further
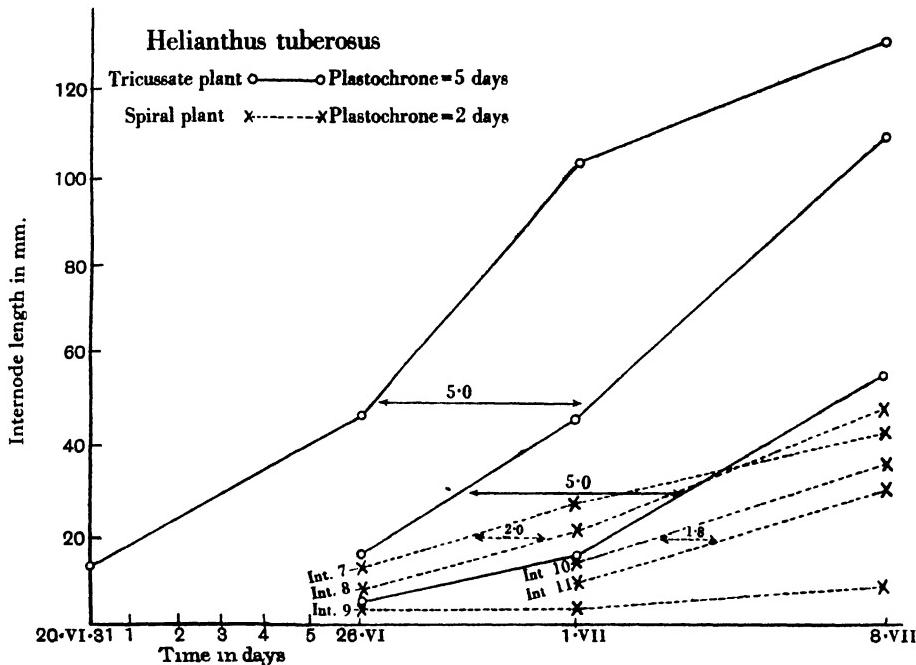

Fig. 10. Comparison of the plastochrone length in *Helianthus tuberosus* with tricussate phyllotaxis (line entire), 5 days, with spiral phyllotaxis (line dotted), 2 days.

supported by comparison of the morphology of such plants, when it is found that in either case the whorls alternate, the growth-units thus extending down through two internodes, regardless of whether two or three units originate at the same level.

The facts observed in *Helianthus tuberosus* were found to apply also to the comparison of 2- and 3-membered whorls in *Veronica* sp., *Fuchsia* sp. and *Diervillea rosea* and also to 3-, 4-, and 5-membered whorls in *Lysimachia* sp.

The results, however, were found to be very different when plants of *Helianthus tuberosus* with spiral phyllotaxis were compared with the whorled types. In the spiral, apparently due to the instability of the system already referred to, the results were very irregular, but where two curves were sufficiently parallel, the plastochrone was determined as approximately 2 days (Fig. 10). Another striking feature of this

comparison was the larger number of internodes which started to elongate in the intervals between two measurements, *i.e.* 3 or 4 in the spiral as compared with 1 or 2 in the whorled types, in a period of about 6 days. In these measurements we are evidently dealing with something more than the time between the origin of two leaf primordia, the plastochrone in its simplest sense, but with the whole period of growth of the shoot unit, which includes in addition to the emergence of the primordium, the longitudinal extension of the region of axis subtending it and which continues to grow with it so long as it forms part of the shoot apex. Comparison with morphology indicates clearly that in the whorled types under consideration, we are not dealing with a paired or "bijugate" spiral, in which members of either spiral have an angular divergence comparable to that in a 2/5 spiral system. Had we been dealing with such a system, the units having the same longitudinal extension as those of the normal single spiral, one would anticipate that the plastochrone for the pair would be the same as that for the single unit in the spiral, the difference being accounted for merely by a larger and more vigorously growing apex. In this case, however, comparison of the spiral and whorled types shows clearly that in the latter the units have undergone a rearrangement and instead of extending down through 5 internodes as in the 2/5 spiral, they now extend through 2 internodes only. So long as this arrangement of alternating whorls is maintained, each unit extending through 2 internodes, it will not be of significance how many members there are in the whorl so far as the plastochrone is concerned, the number being associated with the size and vigour of the apex. In the case of the 2/5 spiral, five plastochrones elapse before a leaf vertically above the first arises at the apex, whilst in the whorled types only 2 whorl-plastochrones elapse before the new whorl of units is formed on the same orthostichy. In such a case it is to be expected that the sum of two whorl-plastochrones should equal the sum of five "single" plastochrones, or the ratio should exist $\dfrac{\text{whorl-plastochrone}}{\text{spiral-plastochrone}} = \dfrac{5}{2}$, which is strikingly in accordance with the measurements made in the case of *Helianthus tuberosus* and very closely in accord with Schüepp's observations in *Helianthus annuus*. In a similar manner, were it possible to compare in the same species the plastochrone in an alternate arrangement with that in a 2/5 spiral, the expected plastochrone ratio would again be $\dfrac{\text{alternate}}{\text{spiral}} = \dfrac{5}{2}$.

Treating the primordia as separate units, as we obviously must do, then two differences characterise the decussate and other whorled systems from the spiral, *i.e.* a varying time interval between successive primordia and a variation of angular divergence from 1/2 to 1/4, which is associated with the variation in the time interval. These are especially necessary to consider since the members of a "pair" do not always appear quite simultaneously or exactly opposite to one another. Very frequently the angular divergence is not exactly 1/2 but approximates more closely to one of the angles more characteristic of a spiral system. Such a divergence in one pair then affects the positions of the pair above so that such a deviation can usually be followed right up the plant. Irregular angular divergences of this kind are

common in many plants, *e.g. Veronica, Vinca, Fraxinus excelsior*, and where it occurs it is often associated with slight differences in the level of insertion of the two members of a pair and differences in the degree of development of the buds in their axils, in fact the association of the smaller bud with the lower leaf of a pair appears to be so constant when systems are examined closely, that it may be taken as the criterion of the lower leaf when this is difficult to determine by a difference in level.

This evidence for the individuality of the members of a pair, coupled with the fact that spiral and decussate systems may often be seen in different parts of the same axis, has led many observers to argue the question of the phylogenetic relations of the two systems. The relation between them from the developmental standpoint would seem to be that both systems represent methods of development of successive growth-units which are associated with individual leaf primordia, and slight variations in the time and place of origin of the next primordium that is characteristic of one system will rapidly lead to a transition to the other. Thus if the members of a decussate pair are less simultaneous, the development of the last of the lower pair will encroach more on the time of appearance of the earliest of the pair above and such competition between the primordia may readily lead also to an angular divergence more characteristic of a spiral system. Transitions from one type to another in the same axis deserve a little fuller examination. In the dicotyledons, embryonic growth of the shoot usually comes to a temporary standstill when two primordia have appeared at opposite sides of the shoot apex and developed so far that they can be recognised as the first leaves or cotyledons, the subsequent leaves being very small or practically undeveloped at this stage. The relatively long halt at about this stage leads to an exaggeration of the time interval between the appearance of these cotyledon primordia and any subsequent ones, an effect especially marked in epigeous seedlings, where the plumule is hardly developed beyond the cotyledons. The cotyledons approximate to a first decussate pair and subsequently, if the shoot apex has any tendency to decussate development, this system is likely to follow immediately in any epigeous seedling, and in practice one finds that decussate seedlings rarely, if ever, pass through a preliminary spiral phase. On the other hand, when an axillary branch arises, subtended by a leaf, the new system does not form under conditions which predispose it to the simultaneous development of the two first primordia and it is very common to find the first few leaves on axillary branches of decussate shoot systems irregularly arranged and showing approximations to spiral arrangement (*e.g. Forsythia, Syringa, Fraxinus, Ligustrum, Jasminum*). Similarly a change in the type of growth is liable to affect the succession of events at the apex and it is not surprising to find that the transition from the vegetative to the reproductive stage is often accompanied by a change from decussate to spiral arrangement (*e.g. Epilobium montanum*).

In the dicotyledons, although the separate primordia are sectorial, when they extend horizontally they may occupy more than half the periphery and if this habit is associated with the insertion of two at the same level, the result may be the development of a meristematic fold which completely surrounds the apex and

subsequently gives rise to a connate pair of leaves as in *Dipsacus, Lonicera*, etc. This union of two adjacent primordia may occur so late or be so little developed towards the point of contact of their two margins, that the line of union is readily recognised, but in more extreme cases the units may undergo common growth for some time and completely enclose within them the basal parts of the next pair of units above. Such a lateral fusion of growth-units was described by Griffiths and Malins (1930), where it is described as though the originally free margins met and fused outside the cuticle covering the intervening units, but the same appearance could be produced, and probably is, by an extension round the axis of the common meristematic fold. The activities of such a continuous zone of tissue will carry up the two original separate primordia, which in serial transverse sections will give the appearance of two free lobes having fused. This coalescence was observed in *Coleus* and *Mentha* and probably occurs throughout the Labiatae; it does not occur in *Syringa* or
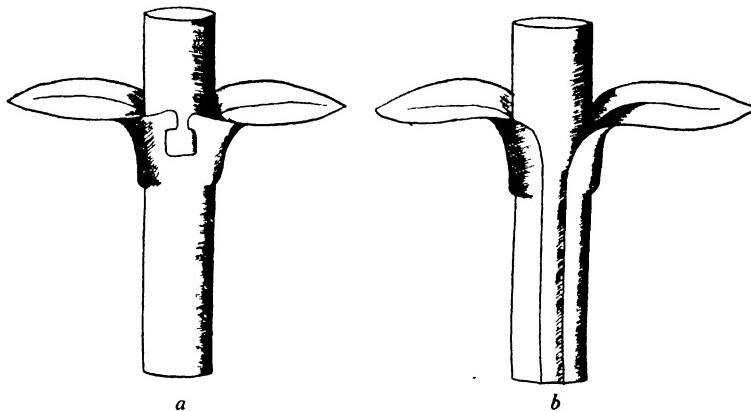

Fig. 11. Diagrams of decussate types. *a*. Labiate type. The primordia fuse laterally around the axis. *b*. Syringa type. The primordia do not fuse laterally.

Ligustrum, in which types the surface of the upper pair of units can be seen in the longitudinal groove between the lower pair (Fig. 11). Lateral union of growing members in this way will be responsible for what Schoute describes as the "binding" of the members of the leaf whorl in *Peperomia* (Schoute, 1925) and the same process has received very careful attention from de Vries in his investigation of the phenomenon of "Zwangsdrehung" or spiral torsion (de Vries, 1892). De Vries follows in this paper the definition of this phenomenon given by Braun, limiting it to decussate or whorled systems in which, as an abnormality, the leaves follow each other in spiral succession instead of in simultaneous pairs or groups, successive leaves remaining attached to one another by one margin. When subsequently the growth-units extend in length their free limbs are unable to separate from one another, owing to the tissue linkage between them at the point of union with the stem and consequently the internodal regions of the units become spirally twisted, in some cases the torsion being so severe as to throw the spiral made by the

coalescent leaf bases into an almost vertical line. It was pointed out by Griffiths and Malins (1930) for *Mentha*, that in plants showing spiral torsion, if one follows a rib corresponding to a growth-unit, down from the insertion of one leaf to the next on the same rib, another spiral line made by leaf scars is crossed midway between the two, thus supplying further evidence of the fact that each shoot unit extends through two internodes.

Thus spiral torsions may be expected to occur in systems which are normally decussate or whorled, if the growth-units appear in regular succession in time without, at the same time, becoming so far removed in space that their margins no longer coalesce at an early stage of development. De Vries (1892) points out further that the succession of primordia at the apex in cases of spiral torsion is actually a sequence of individual primordia in spiral order and that no torsion is visible beneath the apex until the elongation growth begins to take place which is associated with the appearance of internodes.

We may expect to find, then, that spiral torsion occurs when there is a deviation in time of development of the primordia in those decussate or whorled types in which the primordia in a whorl make considerable common growth—that is in the "bound" types of Schoute—whilst in the "unbound" types, *e.g. Fraxinus, Syringa*, deviation from the normal paired arrangement should result in the production of a spiral phyllotaxis. A slight torsion of a rather different kind is often seen in the internodes of certain other decussate plants, *e.g. Clematis vitalba*. This type is unlike the typical spiral torsion in that the direction of twist varies along the same axis. This result may be expected when two primordia are "bound" at a node but not simultaneous in development. If such primordia are practically opposite to one another, the next in the whorl above is about as likely to appear on one side as the other, which would be a very improbable occurrence in spiral torsion, where the single primordia are arising in rapid regular succession and not in a succession of pairs.

The twisted decussate system or "bijugate" type, described by the Bravais brothers, has already received mention and is obviously likely to be produced if there is slight asymmetry of the positions of the members of the first pairs of primordia. Asymmetry in one pair will deflect the position of the next in a given direction, which is likely to remain unaltered in the same axis, successive pairs cutting one another at roughly approximate angles. This type is discussed in detail and illustrated by Church for *Dipsacus* (Church, 1902).

(5) *Whorls of more than two members.*

In discussing spiral phyllotaxis, it was pointed out that more complex systems than the alternate became possible as the size of the apex increased, since the size of the leaf primordium remains constant (being presumably determined by the area which may be supplied by the vascular system of the leaf trace). This is true also of the whorled systems as already mentioned on p. 260. It was observed when determining the plastochrones in *Veronica, Lysimachia*, etc., that the larger the number of leaves in the whorl, the more vigorous was the shoot system. In some

cases a change in the number of members of the whorl may be followed in develop-
ment. In *Hippuris*, as Irmisch (1854) showed, the erect branches arise from axillary
buds on a horizontal branch which bears scale leaves in whorls of three. On the
erect branches the first whorls consist of three leaf primordia which arise in a
definite succession, but in later whorls as the branch develops, the number of leaves
in a whorl increases up to 7-12 as a general rule, but exceptionally as high as 15.
(Schindler (1904) states that a form of *Hippuris vulgaris* is found in northern regions
in which the number of leaves in the whorl is smaller but the individual leaves
relatively broader.) In species of *Peperomia* whorls are found with 3, 4, 5 or 6 leaves
and Schoute has discussed the distribution of these whorls in different species,
attempting to derive them from typical spiral systems (Schoute, 1925). The unequal
divergence between members of the whorl and irregularities in the height of inser-
tion of the various members are in support of such an interpretation, but it is perhaps
safer to conclude that, in these species, the primordia tend to appear in succession
at a divergence less than 1/2 and that therefore, the subsequent disposition of
these leaves on the adult axis reveals many similarities to a spiral system. One very
important question needs discussion in relation to all these plants in which whorls of
"n" leaves are found, namely how many primordia share simultaneously in the
activity of the growing point? It is clear that at least "n" must do so, because these
are formed almost simultaneously, but it is also clear that at least the first member of
the next whorl will have started growth before the previous whorl ceased to grow.
This first primordium of the new whorl would be expected to arise on the opposite
side of the apex to the last of the previous whorl, assuming some time difference
between the members of the whorl to be detectable. But in view of the fact that all
members of the lower whorl will have such a similar growth period, the position of
the new primordium will obviously be influenced by the positions of the two most
nearly below it, with which it should consequently alternate. Extending the same
argument, all the other primordia of this second whorl, arising approximately at
the same time, must appear whilst the lower whorl is still growing and should
alternate with those of the whorl below. In this way we arrive at the conclusion
that $2n$ primordia should be growing at the apex simultaneously, and the members
of successive whorls should alternate. This is really only an extension of the
argument examined more fully in the case of the decussate system and as in that
case, increased differences in the time of appearance of members of a whorl will
lead either to the development of a spiral phyllotaxis or, if the members of the whorl
have coalesced laterally, to spiral torsion.

It is clear that, as in the case of the decussate system, it would be a mistake to try
to derive a whorled system from a particular spiral system or *vice versa*. To illus-
trate this by one particular case, there is no reason to expect that, because the
common spiral systems in the adult plant are 2/5, 3/8, 5/13, etc., the numbers of
leaves in the whorled types should jump from 5 to 8 to 13 with increasing size of the
growing point. In fact, quite to the contrary, we may expect this number to go up
step by step. Considering the introduction of a new member into a whorl with
increasing size of the apex, proportionately a greater increase in periphery is re-

quired to introduce another of the same mass or cross sectional area the smaller the number already present. It is probable therefore, that with increasing size, the numbers of leaves in the whorl will vary more readily so that, whilst dimerous, trimerous and tetramerous whorls are relatively constant throughout an axis, whorls of higher numbers tend to show more variation from whorl to whorl.

IV. SUMMARY.

The problems of phyllotaxis are re-examined from the standpoint of the initial adjustment of the units of shoot growth. The structure of the shoot apex is reviewed in relation to the origin of leaf primordia and it is pointed out that such primordia, once formed, are competing centres of activity and consequently tend to arise as nearly as possible opposite to one another, as exemplified by the simplest case of alternate or 1/2 phyllotaxis.

When more than two primordia are growing simultaneously at the apex, successive ones cannot be exactly opposite, as in this case the position of the new primordium is influenced by all the others which are growing at the same time.

By application of this conception of competing growth centres, it is shown that, as the number of growing primordia at the apex rises, the fraction denoting the new type of phyllotaxis is likely to fall in the Fibonacci series, because analysis shows that these are the only ones which satisfy the requisite spacing of the primordia in relation to the number of time intervals or "plastochrones" separating them in origin.

It is pointed out that systems belonging to the higher fractions in the Fibonacci series are practically impossible to recognise with certainty, unless external observations are supported by anatomy. The case of *Iberis amara* is analysed in detail and it is shown that the 5/13 system could be derived from anatomy.

A rise in the number of primordia growing simultaneously involves readjustment of the units of shoot growth and it is shown that, as the system rises from one fraction to the next, characteristic displacements take place, which vary in direction in different transitions.

The higher systems are usually associated with a wider pith.

It is concluded that the direction of the spiral is determined by the positions of the two first primordia, the genetic spiral being merely an abstraction from the developmental standpoint. This is illustrated by the fact that the direction of the spiral on branches seems to vary irrespective of the spiral on the axis of the same plant.

A decussate system with an angular divergence of 1/2 between members of a pair and 1/4 between successive pairs is regarded as a stable system, provided that the primordia of a pair arise almost simultaneously and time intervals between the pairs lengthen proportionately. This argument is analysed from measurements of the plastochrones in whorled and spiral plants of the same species. Although decussate types often show slight differences in the time and position of development of the members of a pair, this merely demonstrates the tendency to successive

emergence of primordia and not the evolutionary derivation of the system from a theoretical spiral system. If a plant has a tendency to decussate phyllotaxis, this system naturally appears in the seedling because of the temporary halt in the embryonic growth at a stage when two cotyledons occupy the apex. In axillary branches, the symmetry of the phyllotaxis is affected by the subtending leaf and often starts with an approximately spiral system.

In many decussate dicotyledons, the lateral expansion of the primordia causes them to extend over more than half the circumference. In such types, if in abnormal specimens the primordia arise in spiral succession instead of simultaneously, spiral torsion results, the successive primordia remaining "bound" together by one fused margin. Other types of torsion and variations from normal decussate phyllotaxis are also discussed.

In whorled systems of more than two members, the members of successive whorls alternate. Similar torsions and abnormalities to those discussed for decussate types may also occur.

In such whorled types, it is shown that, from the developmental standpoint, $2n$ leaf primordia (or units of shoot growth) must be growing simultaneously, n being the number of members in a whorl.

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EXPERIMENTAL STUDIES UPON THE DEVELOP-MENT OF THE AMPHIBIAN NERVOUS SYSTEM

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(With Twenty Text-figures.)

CONTENTS.

I. INTRODUCTION.

THE desire to obtain a clearer understanding of the nature of forces concerned with the development of normal architecture in the central and peripheral nervous systems of vertebrates has led to many interesting lines of investigation. By reason of the complexities of developmental processes, the interpretation of experimental results has been attended with many difficulties, and our knowledge of the inter-acting morphogenetic agencies lies little beyond the theoretical stage. By direct observation upon developing embryos, through studies upon regeneration, and, in recent years, by the methods of embryonic tissue transplantation and explantation, many interesting and none the less important facts have been discovered. From these facts suggestive theories and hypotheses have emerged. Among these may be cited the original neurotropism theory of Cajal (1892, 1894) postulating the action of chemical substances in the growth and connection of nerves, the dynamic concept

of Strasser (1892) and its modification by Kappers (1917, 1921, 1922), Child (1921) and others, which emphasises the rôle of galvanic phenomena.

Tello (1923) has given a general account of the different neurotropism theories, and his paper contains an excellent account of many important observations which bear upon various aspects of the problems, as well as a thorough bibliography.

Although differing somewhat from each other in details, the theories of Kappers and Child are essentially in agreement in that differences in bio-electric potentials within the developing organism are regarded as being largely responsible for the development of neurone pattern and selectivity within the nervous system. When one considers the question of peripheral selectivity of axones and allied phenomena, many interpretations are difficult unless it is assumed that more or less specific substances (hormones?), which exert specific attraction influences, are at work. This point of view is expressed by Tello; and Child, in his discussion of neurone pattern, says that in the connection of nerves with the peripheral territory, chemotaxis may play a part. Herrick also considers this a factor in nervous differentiation, although he points out that this action is not strongly specific, as is indicated by the fact that nerves will grow and effect functional connections in most atypical places. A discussion of the morphogenetic factors active in differentiation of the nervous system has been given by Herrick (1925), and his paper gives an excellent review of many investigations directed towards a clearer analysis of this important subject.

II. THEORIES OF NERVE DEVELOPMENT.

The controversy which existed for many years regarding the genetic and morphological constitution of the nerve fibre in terms of the cell doctrine served as a powerful stimulus to the invocation of experimental procedures in the study of nerve development. The cell chain theory originally stated by Schwann (1839), and supported by Balfour (1878), Dohrn (1891), Apathy (1897), Bethe (1903), Schultze (1905) and many others, implied that the nerve fibre is the product of a chain of cells which reaches from the centre to the periphery, and that these cells elaborate the fibrillae within their protoplasm much as an embryonic muscle cell secretes the contractile fibrillae. The protoplasmic bridge theory of Hensen (1864, 1868) and its modification by Held (1906, 1907, 1909), and Paton (1907), which received support in the work of Kerr (1904), Braus (1905), Banchi (1905) and others, implied that the nerve fibres are formed out of protoplasmic bridges, existing throughout the embryonic body. Those which serve as conducting elements eventually differentiate into nerve fibres under the influence of functional stimulation, whereas those which do not ultimately disappear.

The outgrowth theory, first stated with remarkable clearness by His (1886, 1887, 1888) and supported by Cajal (1890), von Lenhossék (1892, 1906), Harrison (1901) and others, implied that the nerve fibre is a protoplasmic outgrowth from the embryonic neuroblast, and that this protoplasmic extension continues uninterruptedly from the central ganglion cell to its peripheral termination. Although generally accepted to-day, it was only through experimentation that the outgrowth

theory became definitely founded amongst neuronists in general. Thus not only was there terminated one of the most important controversies in modern biology, but there was established a fundamental principle of nervous development upon which the great progress in organic neurology has been built.

III. EXPERIMENTAL SUPPORT OF THE OUTGROWTH THEORY.

The question of the origin of the nerve fibre was first tested experimentally by Harrison (1904 a, 1906, 1907 a, 1907 b, 1908) and by Braus (1905). Harrison removed the ganglionic crest cells from frog embryos by excising the dorsal half of the cord and found that the larvae developed without sensory nerves and ganglia, but that the motor nerves were present. These were devoid of sheath cells. He showed also that when the ventral half of the embryonic spinal cord was removed, but with the dorsal part of the cord and the ganglionic crest cells intact, the larvae were devoid of motor nerves only. His results indicated clearly that the nerve fibre does not have its origin in the sheath cells, but that it grows from a single ganglion cell, with which it remains in continuity throughout life. A full account of his researches upon the histogenesis of the nerve fibre has recently been published (Harrison, 1924 a). Still more recently Speidel (1932) has added many additional facts upon the myelinisation of the nerve fibre as observed in the living nerve of the tadpole's tail.

In attempts to study experimentally the growth of nerves, Braus (1905) grafted anuran limb rudiments to abnormal positions where they were found to undergo complete differentiation and to acquire nervous connection with the central nervous system of the host. He took advantage of limb grafting as an experimental method for the study of a number of fundamental questions concerning the development of the nervous system. Braus did not believe that the nerves which developed within the transplanted extremity grew in from the host's central nervous system, but that they developed *in situ* and secondarily made connections with the central nervous system of the host. Although all of Braus' experiments were ingenious in type, he endeavoured to support Hensen's (1864) protoplasmic bridge theory, as did also Banchi (1906) who carried out limb-grafting experiments in connection with this same question. Harrison (1907 a) and Gemelli (1906), who also grafted limbs as a means of studying this problem, produced evidence against the Hensen theory, and brought forward valuable experimental support of the outgrowth theory of His.

Whereas the results of Harrison's limb-grafting experiments left little doubt in the minds of most neuro-histologists as to the validity of the outgrowth theory, nevertheless the advocates of the opposing view still demanded more rigorous proof. This proof was soon furnished by the results of Harrison's ingenious and timely tissue-culture experiments (1910) in which he explanted neuroblasts of frog embryos into clotted lymph outside of the body, and observed with the eye the developing nerve fibre as a protoplasmic outgrowth from a single ganglion cell. These results and their corroboration by Burrows (1911), and Lewis and Lewis (1911), definitely and beyond the slightest shade of doubt established the "neurone" as the genetic and morphological unit of the vertebrate nervous system, and there

was thus given to biology a definite concept of nerve growth so necessary to the advance of neurology as a science—not to speak of a new method in biology which is employed extensively in every research institution which aims to study cellular physiology.

Although it seemed clear from the experiments on the explanation of primitive ganglion cells that the initial outgrowth of the nerve fibre can proceed independently of any functional requirements on the part of the end-organ, no especial attempt was made in these experiments to study the effects of normal organic stimuli upon the extent of neuronic growth and differentiation. The results of experiments directed towards the solution of this question (Braus, 1906; Dürken, 1911; Shorey, 1909, 1911; and Burr, 1916a) indicated that in the absence of certain peripheral areas, the nerve centres normally supplying those regions undergo hypoplastic development—supposedly from the lack of a peripheral growth stimulus which normally activates their complete development. Shorey, who extirpated the fore-limb rudiments of *Amblystoma* and the chick, claimed to have found marked deficiencies in the peripheral nerves as well as in the ventral horn areas. Recently similar claims have been made by May (1930) upon frog embryos following hind-limb excisions. Shorey (1911) explanted neuroblasts in a variety of tissue culture media, and claimed that axon growth ensued only in those cases in which beef extract (metabolic products of muscle) was supplied in the culture medium. As a result of her observation she concluded that motor nerve growth is not only entirely dependent upon the presence of muscles, but that no neuroblasts can differentiate unless under the stimulus supplied by the functional end-organ or under the influence of its metabolic products.

Burr (1916a) in his original experiments involving the excision of the nasal placodes in *Amblystoma* embryos concluded that after an early period of self-differentiation in Roux's (1885) sense, the hemisphere undergoes a hypoplastic development in the absence of the nasal placode, supposedly from the lack of the necessary functional stimulation normally travelling from end-organ to nerve centre. Although Burr has more recently (1920) altered his view regarding the rôle of functional activity as a factor in nervous proliferation, it was this and other early experiments upon this question that prompted the author to investigate this matter by means of grafting limb rudiments to a new locality. This was done so that not only the effects of the absence of a peripheral structure upon nervous development could be studied, but also to test whether by overloading the periphery at a given region the corresponding peripheral neurones could be induced to undergo hyper-plastic growth in response to the added needs placed upon them.

Whereas many interesting facts bearing upon the problems of proliferation of nerve cells have been obtained from the limb-grafting experiments, they will be presented and discussed in a later section—and for the present we shall consider other responses of growing nerves to experimental alterations in their peripheral field.

IV. EXPERIMENTAL ALTERATION OF THE DIRECTION
OF GROWTH IN SPINAL NERVES.

The original limb-grafting experiments consisted in the excision of the anterior limb rudiment of *Amblystoma* embryos and its reimplantation at distances varying from one to seven segments caudal to the normal position (autoplastic grafts). It had been shown previously by Harrison (1915) that the rudiment in this form is located ventral to the third, fourth and fifth somites (Fig. 1), and that when it was grafted (homoplastically) to a new environment it would undergo complete structural differentiation (Fig. 2) and acquire nerves from that region of the host to which it was grafted. This fact had been demonstrated previously by Braus (1905), Banchi (1906), Gemelli (1906) and Harrison (1907a) for anuran embryos.

Fig. 1. Drawing of *Amblystoma* embryo in Stage 29 The circle ventral to the pronephros (*PN*) indicates the position of the fore-limb rudiment *SO* 1, first somite, *SO* 3, third somite, *GS*, gill swelling ∠ 10.

Although observations on the segmental nerve supply and the functional responses of the transplanted limbs have been reported previously (Detwiler, 1920b, 1926a), it may not be redundant here to state that limbs which were grafted from one to three segments caudal to the normal position in the autoplastic series, usually became supplied by the original brachial nerves (third, fourth and fifth spinal nerves). Limbs grafted four and five body segments caudal to the original site frequently received the fifth nerve, and in addition, a varying number of nerves originating


Fig. 2 Larva of *Amblystoma punctatum* (63 days old) showing right anterior limb grafted the distance of five segments caudal to the normal position. ×2.

caudal to the brachial region of the spinal cord (Table 1). These cases, as a whole, showed that the segmental nerves contributing to the limb plexus arise mostly from higher levels of the cord than that occupied by the graft, and, that in forming nervous connections with the limb, such nerves grew considerable distances out of their normal pathways (cf. *a* and *b* Fig. 3)—particularly the original brachial nerves.

A study of the functional responses of the grafted appendages showed that

typical co-ordinated movements ensued only in those cases where the limb was wholly or in part connected with the normal brachial region of the cord. Connection of the limb with the fifth nerve or even a branch was sufficient to insure co-ordinate activities. When the grafts were placed so far caudally as to receive their nerve supply from cord segments entirely caudal to the brachial region, the function was very limited in spite of a rich peripheral nerve supply. In such cases the limb responded reflexly to mechanical stimulation, and in a few instances "spontaneous

Table I. *Showing the segmental nerve contribution to the right fore-limb of* Amblystoma *when transplanted. A. One to three segments anterior to the normal position* (Series AA 1 S, AA 2 S). *B. One to five segments caudal to the normal position* (Series AS 1, AS 2, etc.).

	Series		Cases	No. of segments anterior to normal position	No. of segments caudal to normal position	Segmental nerve contribution 1 2 3 4 5 6 7 8 9
		Normal	1			3 4 5
	AA 1 S		3	1		3 4 5
			24	1		2 3 4
A	AA 2 S		15	2		2 3 4
			21	2		2 3 4
			25	2½		2 3 4
			73	2		2 3 4
			78	2–3		1 2 3
	AS 1		12		1	3 4 5
			17		1	3 4 5
	AS 2		5		2	3 4 5
			12		2	3 4 5
	AS 3		9		3	4 5 6
			18		3	4 5 6
B	AS 4		12		3	4 5 6 7
			13		3½	4 5 6
			24		4	5 6 7
			26		4	5 6 7
			30		4	5 6 7
	AS 5		25		5	5 6 7 8 9
			27		5	6 7 8 9
			30		5	6 7 8 9

movements" occurred, but in no case were they co-ordinated with the activities of the intact limbs. These observations showed, then, that nervous connection with the general brachial region of the cord is necessary for the performance of co-ordinate activities in grafted limbs. This was substantiated further by experiments in which the anterior limb was left intact and a supernumerary rudiment was grafted three or four segments caudal to the normal. In the majority of such cases the supernumerary limb became innervated by the sixth, seventh and eighth nerves and failed to exhibit co-ordinate function. Occasionally, however, the graft received

all or a branch of the fifth nerve in which case co-ordinate movements ensued (Detwiler and McKennon, 1929).

The remarkable caudal growth of the brachial nerves to the limbs when the latter were shifted four and five segments caudal to their normal position, indicated the presence of an attractive influence on the part of the developing appendage. The non-specificity of this attraction between limb muscles and limb nerves was clear from the fact that nerves arising from post-brachial segments of the cord would exhibit similar caudal growth to the limb. The characteristic growth response of the nerves was attributed to the fact that those nerves lying anterior to the position of
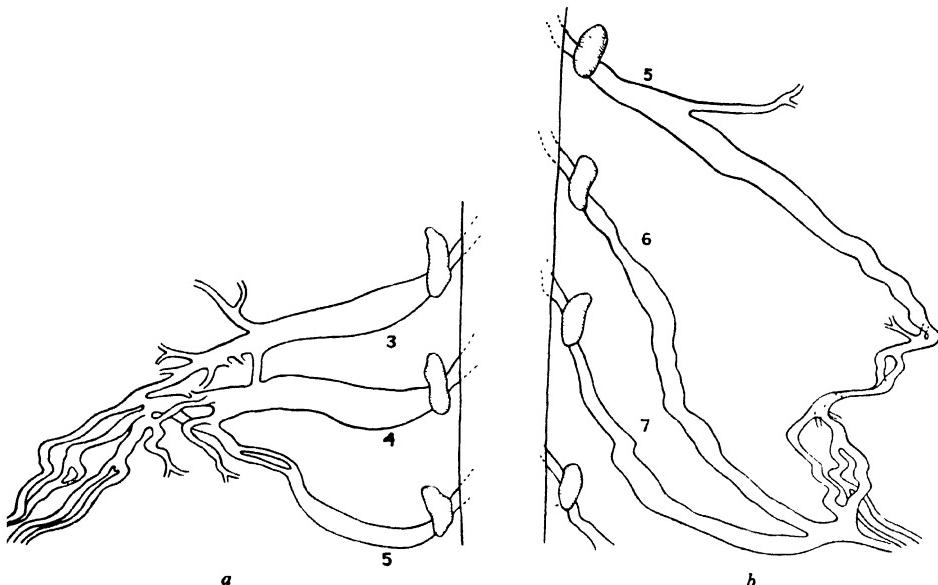

Fig. 3. (a) Graphic reconstruction of the normal left brachial plexus in an *Amblystoma* larva 68 days of age (case AS 4-26). ×25. (b) Graphic reconstruction of the right brachial plexus in *Amblystoma* larva AS 4-26 (68 days after operation), showing segmental nerve supply to the right anterior limb which was grafted the distance of four segments caudal to the normal position. ×25.

the graft are more advanced in their outgrowth than are those corresponding to the level occupied by the graft and are thus in a condition to be more strongly attracted.

It is apparent that spinal nerves are influenced in their caudal growth by the caudo-lateral elongation of the developing myotomes which presumably assist the nerves in this direction, yet such mechanical assistance could not possibly explain why nerves make connections with the shoulder and limb muscles when they reach this region unless some strong attractive influences are present.

That this remarkable growth of the spinal nerves to grafted limbs was not due to mechanical growth influences was demonstrated by a second set of experiments. In these the limb was grafted several segments cranial to the normal position so as to lie in the gill region—a most unfavourable site for normal limb differentiation by

reason of the strong potencies of this region to develop gills. In many such cases one or more of the brachial nerves grew cephalad the distance of several segments (Fig. 4, cf. Fig. 3 a, Table I) to make connection with the grafted limb (Detwiler, 1922). Since the anterior growth of the nerves under such conditions has taken place against the general mechanical influences which tend to direct them caudally,

it is indicated still more strongly that there is some strong attractive influence exerted by the differentiating limb upon nerves arising from the general brachial region of the cord. That such forces are operative through only a relatively short period of time was shown by a third set of experiments in which the limb bud was excised in the tail-bud stage (Stage 29) and the embryo was allowed to develop until the nerves had grown out to the periphery. A limb bud was then grafted four segments caudal to the normal limb site, and in most cases studied subsequently it was found that the graft received its nerves from segments of the cord corresponding to the level occupied by the limb, whereas the normal brachial nerves (third, fourth and fifth) grew out to the limbless area (Detwiler, 1924 a).

Fig. 4. Graphic reconstruction of segmental nerve supply to transplanted fore-limb in case AA 2 S– 25 (50 days after operation). The limb occupies a position approximately 2¼ segments anterior to the normal. *A* indicates level of grafted limb; *B* designates normal limb level. ×25.

Still further evidence demonstrating the attractive influence of the differentiating limb upon outgrowing spinal nerves was obtained from another set of experiments (1925 a). Here the right anterior limb rudiment was excised and grafted the distance of four body segments caudal to the normal position. Under such conditions one or more of the brachial nerves typically grow caudal to the heterotopic appendage. In these experiments, however, the graft was made so that regeneration of a limb in the normal region might ensue. This was accomplished by not grafting the entire rudiment and by not cleaning nor covering the wound, which is usually necessary to prevent regeneration (Harrison, 1915), since this system has been shown repeatedly to be an harmonic equipotential restitution system (Harrison, 1917, 1918; Detwiler, 1920 b; Nicholas, 1924; Swett, 1926). Following this procedure there developed a limb in both the heterotopic and the orthotopic positions (Fig. 5) and in such a way that initial limb development in the former position was in advance over that in the latter region. Such a situation would theoretically set up two centres of attraction upon the developing brachial nerves. In many cases studied with two limbs and in some cases three (since the graft frequently underwent reduplication) the normal brachial nerves were distributed to all the appendages—although such distribution did not always involve any peripheral communication of the nerves (Fig. 6 a). Observations on the function of the grafted limbs showed that the homologous muscles always contracted synchronously and with the same degree of intensity. Not only was this observed unfailingly with the eye, but an analysis of slow-motion cinematographic records bears out this statement.
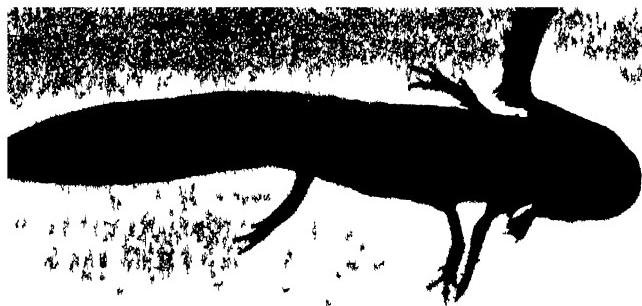
Fig 5 Photograph of *Amblystoma* larva AS 4–39 (53 days after operation) The right anterior limb rudiment was grafted (with inverted orientation) the distance of four body segments caudal to the normal position A limb regenerated in the orthotopic position ×2


b

Fig 6 Diagrammatic plan of the segmental nerve contribution to a transplanted limb (*TL*) and to a regenerated limb (*RL*) in *Amblystoma* larvae (*a*) case AS 4–13, 41 days after operation, (*b*) case AS 4–39, 53 days after operation The normal brachial nerves are shown in Fig 3*a*

Disregarding for the moment our inquiry into the possible mechanism respon
sible for this remarkable phenomenon of homologous function, it is none the less
striking to note that under the conditions of the experiments all of the brachial
nerves were heavily overloaded and yet many cases exhibited the full quantity of
activity in all of the limbs. This is particularly striking in certain cases where three
limbs were present and in which the fifth nerve, in addition to supplying a branch
to the regenerant, innervated both heterotopic limbs and was responsible for their
co-ordinated movements, as will be shown later. In consequence of this heavy
peripheral overloading, the distal portions of the nerves became enormous in size—
thus indicating that functional regulation was accomplished by repeated division
of the axons, since it will be pointed out subsequently that there was no evidence to
indicate a hyperplasia of motor neuroblasts.

The phenomenon of homologous, synchronised function in corresponding muscle
groups of grafted and normal appendages was observed also by Weiss (1923, 1924,
1928) in cases where fully differentiated limbs (fore and hind) were grafted close to
the normal in larvae of *Salamandra maculosa*. In making the wound for the inser-
tion of the limb Weiss cut some of the nerves to the normal limb. These regenerated
into those muscles of the orthotopic limb rendered nerveless by the nerve section,
as well as into the denervated muscles of the extraneous limb. Weiss pointed out
the important fact that analogous movements in corresponding extremity muscles
are not correlated with specific nerve regeneration to homologous muscles. Since
there occurred "at random" regeneration to the various muscles in both limbs, it
became evident that the homologous function could not be explained upon any
specificity in structural pathways. This was evident since a single axon during
regeneration might send branches to non-homologous muscles in the two limbs.
Therefore, when that particular axon carried a nerve impulse, it was obvious that
not all the muscles responded which were in connection with it. Otherwise it
would produce movements which were not homologous, a functional response
which did not occur. In order to seek an explanation for his results, Weiss hypo-
thesised the so-called "Resonance" principle in reflex activity which assumes that
a muscle does not respond to all excitations but only to those which are proper for it.
The nerve is regarded as being able to convey different excitation, and whether or
not a muscle will respond depends upon whether it receives the specific excitation
to which it is attuned. In other words it acts like a "Resonator." According to
Weiss, 1926, p. 245: "The central co-ordination consists in combining the excita-
tion for those muscles that are to function at a certain given movement. All these
component excitations are conveyed together in the same manner through all the
fibres of the motor part of the limb level of the spinal cord to the periphery where
the particular muscles respond to their respective specific components of the whole
excitation complex. Thus, while all components of an excitation are conveyed to all
muscles, only such muscles will respond for which a proper specific component was
contained in the whole excitation. This accounts for the fact that the peripheral
co-ordination is always in accord with the central co-ordination without in any way
depending upon a definite configuration of conduction paths."

Apparently it is the end-organ in the muscle which is responsible for the sorting out of specific excitations, since, before they are developed and functioning, the excitation specificity of the muscle is not observed (personal communication).

Recently Weiss (1931 a) has strengthened his concept by new experiments involving the transplantation of supernumerary muscles in the metamorphosed toad (*Bufo viridis*). He implanted to fixed skeletal points, and under tension, various single individual leg muscles from one side of the body to the other and led into the implanted muscle a branch of the lumbo-sacral plexus. After the grafted muscle had become functional, it and its homologous intact leg muscle, along with a non-homologous leg muscle, were attached separately to a kymograph. When these three muscles responded to tactile stimulation of the animal, the grafted and homologous intact muscles contracted at the same time and with approximately the same degree of intensity, as indicated by the height of the tracing on the smoked drum, whereas the contractions of the non-homologous muscle showed no correspondence with the other two. Weiss shows many myograms to illustrate this characteristic response, which ensued regardless of what nerve branch was introduced into the grafted muscle or of what combinations of muscles were employed. The phenomenon of analogous synchronised function in homologous muscles was the same here as was observed in grafted salamander limbs and in the supernumerary limbs of an adult frog obtained from nature (Verzár and Weiss, 1930).

As important as Weiss' facts are and as fascinating as his theory is, one is confronted with certain difficulties in accepting without some reservation his principle of reflex activity. Supernumerary limbs, when grafted several segments caudal to the normal, fail to execute co-ordinate and homologous function (Detwiler, 1920 b). One might assume that this failure is due to the fact that they are connected with those spinal nerves (six, seven and eight) which carry excitations for trunk muscles, for no matter how long post-brachial nerves are in connection with grafted limb muscles, they are incapable of bringing about homologous movements, yet when the anterior limb rudiment is grafted on to the head in place of the ear vesicle (Detwiler, 1930 a) such limbs exhibit a high degree of function which is co-ordinated with the activity of the jaw muscles. The cranial nerves involved, therefore, are capable of carrying excitations for limb muscles, whereas spinal nerves, caudal to the brachial region are not. Whether the grafted limb is a rudiment or is a fully developed appendage makes no difference in the results, for Carpenter (unpublished) has shown recently that a limb which has been functioning in its normal position for several months during larval life can be grafted to the head, and after cranial nerves regenerate into the graft it exhibits the same type of co-ordinate function as does a limb rudiment when grafted to the same position in the embryo. The function of such limbs is not transient, since it has been observed in my laboratory to continue in axolotls for a period of nearly four years. Interesting observations in this connection have been made by Lovell (1931) who grafted supernumerary hind-limb buds close to the normal in *Amblystoma* larvae. Those and only those which receive a contribution from the normal lumbo-sacral nerves (15, 16, 17) exhibit definite homologous movements, but in some cases where the heterotopic appendage was

supplied by nerves anterior to the lumbo-sacral nerves (*e.g.* thirteenth and fourteenth), it exhibited marked functional activity when the jaw muscles were in action, yet during this period all the remaining limbs and the body were inactive.

Here again we see that those spinal nerves which are not normally involved in a limb plexus, even though adjacent to it, are incapable of producing the necessary excitations to bring about co-ordinated or homologous movement. Whereas Weiss' hypothesis is an ingenious one, and whereas his experimental facts appear to give undoubted support to it, there are still other experimental results which do not appear to be so readily interpreted in terms of his principle. Yet there is no doubt that his valuable contributions will stimulate a great deal of critical investigation of reflex physiology.

It has been pointed out earlier that grafted limbs can exhibit co-ordinate activities only when they receive some contribution from that region of the cord which normally supplies nerves to the limb. Grafted fore-limb rudiments show co-ordinated movements when supplied by the fifth, sixth and seventh spinal nerves —the fifth of which is a brachial nerve. Whether the sixth and seventh are capable of sustaining such activities after their initiation through the fifth has been tested experimentally (Detwiler and Carpenter, 1929). In embryos of the tail-bud stage, the right anterior limb rudiment was grafted caudally four body segments. Under these conditions the limb is usually supplied by the fifth, sixth and seventh nerves. In such cases as developed well-defined co-ordinated activities in the grafted limb, a second operation was performed which consisted in severing individual spinal nerves supplying the graft. This was done on larvae ranging in length from 30 to 40 mm. The results showed that co-ordinated movements in a limb which is supplied by the fifth, sixth and seventh nerves always cease when the fifth nerve is severed, even though such movements have been carried out over a period of several months. The intactness of the sixth and seventh, after interruption of the fifth, is capable of bringing about simple reflex responses to tactile stimulation, but these nerves play no rôle in the co-ordinating process. When the fifth nerve is left intact, the sixth or seventh nerves or both may be severed without any loss of co-ordinated function. These results furnished additional evidence to show that the normal brachial correlating mechanism cannot be prolonged caudally in the cord. This mechanism is built up within the third, fourth and fifth segments, and up to the present time we have been unable to alter its limits by imposing new functional requirements at the periphery.

The atypical growth response of brachial nerves to grafted limbs naturally raised the question as to whether the attraction which the limb buds exert upon such nerves is an expression of any specific developmental relationship, or whether it is to be regarded only as a growth response towards a centre exhibiting high physiological activity. In other words, if other rapidly differentiating systems are grafted to similar positions, will they attract nerves from these same spinal levels? In order to test this, the anterior limb rudiment was removed in the embryo (tail-bud stage) and a nasal placode from another embryo was grafted the distance of four segments caudal to the normal limb position (Detwiler, 1928a). It will be remembered that

Experimental studies on amphibian nerve development

limbs grafted caudally to this same position usually became innervated by the fifth, sixth and seventh spinal nerves (Fig. 3b). In some cases the nasal placode alone was used; in others the optic cup was grafted along with the nasal rudiment (Fig. 7). In a number of cases studied following this operation it was found that the fifth nerve, which typically grows somewhat anteriorly to the limb, grew caudally a considerable distance to the region of the differentiating olfactory organ (Fig. 8a, cf. Fig. 3a). The sixth nerve also grew towards the differentiating placode, or towards the more caudally situated grafted eye. In several cases in which a limb regenerated in the orthotopic position, the fifth nerve bifurcated—one branch going cephalad to the regenerant and the other caudally to the placode—thus exhibiting a growth response exactly similar to that shown when it supplied a supernumerary grafted limb and a regenerating orthotopic one (Fig. 8b, cf. Fig. 6b).
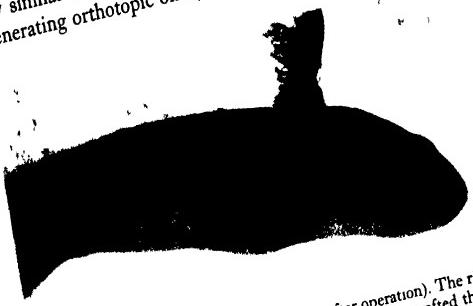

Fig. 7. Photograph of *Amblystoma* larva LETE–12 (38 days after operation). The right anterior limb rudiment was excised and an optic cup with adjacent nasal placode was grafted the distance of four body segments caudal to the original limb position. × 2·7.

That the motor roots of the fifth and sixth spinal nerves were found to grow towards a grafted nasal placode, and in one case actually to penetrate the organ, offers strong evidence that attractive, non-specific influences may be set up by a rapidly differentiating system, even though it is strange to the nerves involved. In these cases we find that a sense organ attracts nerve fibres which typically go to muscle. In these experiments it was found that the attraction exerted by the nasal organ was greater than that exerted by the eye, although in several cases one or two spinal nerves were found to grow out to the eye and some of the fibres actually ended in the loose tissue surrounding it, rather than to make connection with the muscle which represents their normal end territory. Whether the attraction exerted by the nasal organ is due to the organ as a whole or due only to the differentiating nervous elements cannot be stated definitely.

Wieman and Nussmann (1929) transplanted the limb three and four segments caudal to the normal position, and then grafted an optic vesicle into the original limb site. The brachial nerves were found to grow out towards the implanted eye and, in a few cases, some of them grew caudally to the limb. They concluded that the developing eye tissues produced no marked, if any, attractive influence upon the

growing brachial nerves, since the nerves after reaching the bulb were deflected. Nussmann (1931) excised the limb and substituted an optic vesicle. Under these conditions where there was no limb present in the vicinity of the eye, the brachial nerves grew out towards the grafted eyes, but passed beside them and ended in muscle.

It is not entirely clear from the results of Wieman and Nussmann whether the optic vesicle attracts or not—since when the limb bud is removed and no other organ is substituted for it, brachial nerves will grow out to the remaining peripheral musculature at the old limb site. Viewing their experiments along with many
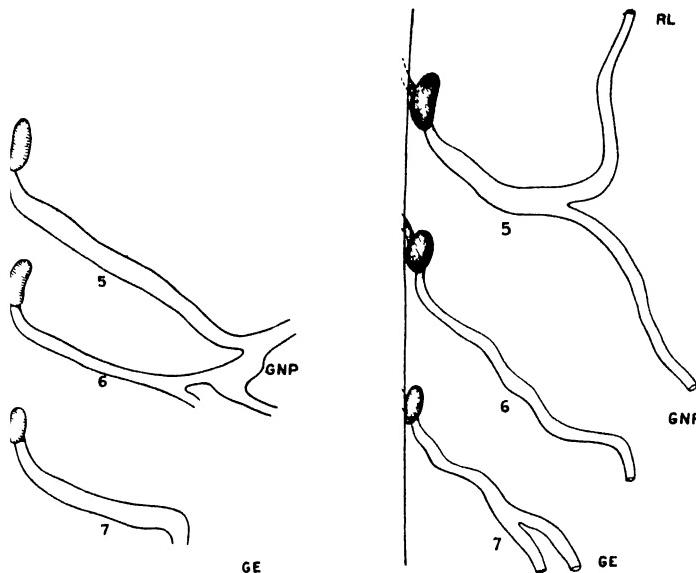

Fig. 8. Graphic reconstructions showing the direction of growth of the fifth, sixth and seventh spinal nerves in two experimental animals (×25), in each of which the right anterior limb was excised, and a nasal placode (*GNP*) and an optic cup (*GE*) were grafted 3–4 segments (*a*) and 4 segments (*b*) caudal to the limb region (see Fig. 7). The normal course of the fifth nerve is shown in Fig. 3*a*. (*a*) Case LETE-13, (*b*) Case LETE-46. In (*b*) a limb (*RL*) regenerated in the normal position.

others of similar type, it would seem that the eye probably does exert some very general non-specific influence, but that this is not nearly so strong as that exerted by the nasal placode.

Although there are many points in all these experiments which need clarification, it appears established that developing systems which normally have no relation whatsoever to spinal nerves will attract these nerves towards them. In this respect the results appear to bear out those of Hoadley (1925). He grafted developing chick mesencephalon together with somitic tissue to the chorio-allantoic membrane and found that nerve fibres, which are normally never related to muscle (visual correlation fibres) grow out for considerable distances from the mesencephalon into the

differentiating muscle. He found that these nerve processes would also penetrate other tissues such as mesonephros and cartilage. Hoadley has discussed these reactions in relation to the theories of Strasser (1892), Kappers (1917) and Child (1921) and is inclined to view the attraction as a dynamic (galvanic) phenomenon rather than one of chemotaxis.

That spinal nerves within the embryo may be attracted by developing tissues with which such nerves normally have no relation whatsoever indicates that in normal development growing nerves are attracted towards peripherally developing tissues. That such attractions are of a very general nature is seen not only from these experiments, but also from those of Hoadley. The fact that nerves will be attracted by strange tissues without actually making connections (eye and nasal placode) appears to strengthen the idea that in the growth and connection of peripheral nerves with various organs in development, two groups of forces are acting. The first of these appears to include forces of very general nature, which may actually prove to be galvanic as suggested not only in various theories alluded to previously, but in the experiments here described. Such forces seem to be responsible for the general direction of growth towards the end-organs. In this connection Bok (1917) has pointed out that connection between certain muscles and sometimes widely distant places of the central nervous system has to be explained by the fact that the contraction of muscle (which precedes the formation of nerve roots) acts tropistically upon the central fibres. Kappers suggests also the probability that, in embryos, the proliferation of muscle may activate irradiations of nervous currents from the spinal cord. In this connection he cites the observations of Herrick and Coghill (1915) upon the development of reflex mechanisms in *Amblystoma*, in which they showed that the first outgrowths to the myotomes are collaterals from intraspinal motor tracts. Coghill (1926) has more recently shown that nerves which eventually go to the limb at first grow out to and spread through the myotomes, and only later go on to the limb. Thus there is developed a nervous mechanism responsible for co-ordinating the early limb movements with the trunk musculature. In spite of this normal developmental relationship it will be shown later that the presence of myotomes is not necessary for brachial nerves to grow out to the limb.

Although bio-electric conditions set up as a result of high physiological activity (Child, 1921) may be responsible for the direction taken by outgrowing axons towards a field of rapid proliferation, the matter of intimate connections of the nerves with the end-organs seems to fall more under the influence of a second set of forces. These seem to be more specific in nature and the postulation of the reaction of nerves to chemically specific substances seems necessary in the present state of our knowledge, to understand properly peripheral nerve selectivity during normal development. This point of view has already been expressed by Cajal, Child, Kappers, Tello and others.

In connection with the matter of abnormal nerve growth to limbs, Hamburger's (1927, 1928, 1929) experiments are of especial interest. He carried out unilateral extirpations of that region of the embryonic spinal cord of frog embryos which normally develops the lumbo-sacral nerves. A number of cases were obtained in

which the plexus on the operated side failed entirely, but in others he found that a nerve of varying size grew across the midline from the contralateral normal plexus and supplied the denervated limb (Fig. 9), thus indicating a strong attractive influence of the developing limb upon growing nerves of the opposite side of the frog.

The abnormal growth direction of spinal nerves to such an organ as the nasal placode suggested the experiments in which the tail bud, which was selected as a region of rapidly growing tissue, was grafted to the side of the body, four segments caudal to the limb region in embryos from which the limb rudiment had previously been excised. These grafts, although growing rapidly, failed to exert any attractive influence on the growth of the host nerves, such as did the nasal placode and eye, in spite of the fact that the tail bud must be considered as a region of high physiological
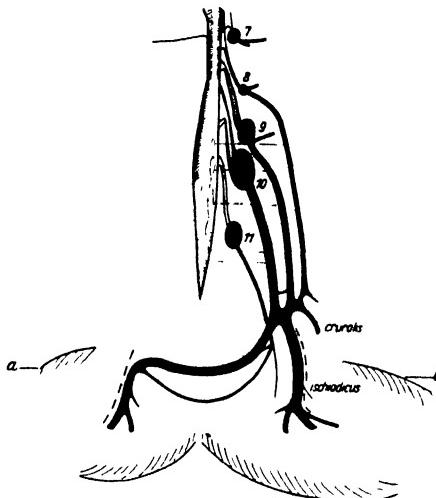

Fig. 9. Reconstruction showing absence of right hind-limb nerves of the frog. The right lumbo-sacral region of the cord was excised in the early embryo. A large nerve from the normal left lumbo-sacral plexus has crossed the mid-body line to supply the right leg. After Hamburger (1929).

activity. Although it is impossible to say definitely, it appears as though the failure of the growing tail bud to attract host spinal nerves was due to the fact that the differentiating muscles in the growing tail became adequately supplied by nerves arising from the isolated piece of cord which developed in the graft. The muscles developing in the tail represent normal end territory for the nerves growing out from the spinal cord within the graft. Moreover, the body musculature of the host represents the normal end territory for host spinal nerves. So we have here essentially two independent neuromuscular mechanisms which develop in normal fashion without any influence one upon the other. It could hardly be expected that host nerves with a longer distance to grow should penetrate the developing tail muscles, even though some attraction might be exerted, when the latter muscles are in relation to the tail nerves which have only short distances to grow to innervate them. Whether the growth of nerves from the isolated cord into the developing tail

muscles sterilises them against additional innervation is an open question. Harrison (1910*a*) called attention to the view that when a cell is ripe for innervation, the first nerve reaching it will make connection and change its nature so that other nerves subsequently reaching that vicinity cannot make connection. In this sense the reaction is analogous to the fertilisation of the egg by a sperm. If the susceptibility of muscle to additional motor innervation is terminated normally when its initial motor connections are established, such stabilisation, however, does not permanently preclude the possibility of a muscle being functionally innervated by other nerves. This has been shown experimentally by Elsberg (1917) and others. Experiments, also, upon the hyperneurotisation of mammalian muscle have been described by Stookey (1922).

In order to study this matter experimentally in the embryo, Rogers (1929, 1932) and Yamane (1930) grafted the brachial region of the spinal cord with various orientations adjacent to the developing anterior limb. Yamane worked with *Amblystoma mexicanum*, and grafted the isolated unit of cord between the limb rudiment and the developing gill mound so that the long axis of the graft lay dorso-ventral. Yamane failed to find any tendency for the nerves coming from the graft to innervate the developing limb muscles. Although they grew out from the cord, they ended in other neighbouring tissues. Rogers grafted accessory units of spinal cord in four positions with respect to the limb rudiment, viz. dorsal, ventral, anterior and posterior. Dorsally situated grafts generally developed nerves which supplied the limb, those occupying an anterior or posterior position only occasionally innervated the appendage, whereas those in the ventral position failed entirely to contribute nerves to the limbs. In many cases, the limb, in addition to being innervated by the grafted cord, received a rich nerve supply from the normal cord. Consequently the limb muscles were supplied by two cords (normal and grafted). The behaviour of the limbs revealed this condition of affairs even before sectioning, for the limb would frequently undergo tetanic contractures lasting for a given period following tactile stimulation. Again, the limb would function co-ordinately with the opposite normal one. Hence it was clear that nervous discharges were reaching it from both cords. In a physiological study of these cases Rogers attached the limb muscles to a kymograph and measured the height of contractions following impulses arising from both intact and supernumerary cords as initiated by a faradic current. These he found to be similar in many cases, thus showing that the muscles were supplied by the grafted as well as by the normal cord. Thus Rogers has shown both on structural as well as physiological grounds that hyperinnervation of a given embryonic muscle is possible. Whether a single muscle cell receives a nerve fibre from both sources is still unsettled, although it is hoped that the improved silver technique which Rogers (1931) has devised will eventually decide this matter.

V. DEVELOPMENTAL RESPONSES OF SPINAL GANGLIA TO ALTERATIONS IN THE PERIPHERAL FIELD.

As was stated previously, it was in connection with an investigation of the possible rôle of functional activity upon neurone development that the writer first carried out limb-grafting experiments in the embryo (1920a). This was done to study not only the effects of the destruction of peripheral areas (limb ablation) upon neurone development, but to test whether or not by overloading the periphery at a given region, the corresponding peripheral neurones could be induced to undergo hyperplastic development in response to the added needs placed upon them.

The most instructive cases were those in which the anterior limb rudiment was excised and reimplanted the distance of four body segments caudal to the normal position. It will be remembered that limbs so placed became innervated typically by the fifth, sixth and seventh spinal nerves, the fifth of which is a normal limb nerve. Microscopical examination of such cases showed that the strange segmental nerves (sixth and seventh) contributing to the grafted limbs were larger than the contralateral nerves, which, of course, had no connection with a limb. This enlargement was found to be due to a hyperplasia (approximately 40 per cent.) of the sensory neurones. The evidence of sensory hyperplasia was not only suggested by the obvious differences in the sizes of the spinal ganglia (Fig. 10c, d) and the posterior roots, but was estimated quantitatively by making a numerical count of the sensory ganglion cells. Excision of the limb rudiment was found also to result in a 50± per cent. hypoplastic development of the sensory neurones of the limbless area (Fig. 10a, b). From the fact that the percentage of volume reduction of the posterior roots was relatively greater than in the ganglia (Detwiler, 1924b), the results indicated that, in addition to a hypoplasia, a slight atrophy of the afferent neurones had also ensued. This characteristic developmental response of the spinal ganglia to diminution and to increase in the peripheral territory has been observed unfailingly over many years. These results have been supported chiefly by Carpenter (1932, 1933), who has successively grafted older limb rudiments; by Schwind (1931),


Fig. 10. Reconstruction models. (a) Left third and fourth spinal ganglia which are connected with the normal limb. (b) Right third and fourth spinal ganglion which have undergone marked hypoplasia resulting from excision of the right fore-limb in the embryo. (c) Normal left sixth and seventh spinal ganglia which normally have no connection with the limb. (d) Enlarged right sixth and seventh spinal ganglia which have undergone hyperplasia when in connection with a transplanted limb. ×40.

who grafted *A. tigrinum* limb rudiments heteroplastically in place of those of *A. punctatum*; by Balinsky (1927), who studied the ganglia of spinal nerves supplying "induced" extremities; by Wieman and Nussmann (1929), and by Nussmann (1931). These responses have not been limited to spinal ganglia, for it has been

shown that when limb rudiments are grafted to the head, the cranial ganglia likewise undergo hyperplastic responses (Detwiler, 1930a).

When spinal nerves are brought into relation with a transplanted limb they not only have a greater integumentary area to supply, but by reason of the added musculature (limb and shoulder muscles) it is reasonable to expect that there should be an augmentation in the number of proprioceptive fibres to the muscle. Since it is impossible to distinguish between exteroceptive and proprioceptive neurones in larval ganglia, it was impossible in the limb experiment to determine what percentage of cell decreases or increases was due to alteration in the surface area of skin and how much to alteration in the volume of muscle. Consequently, indirect means were taken to investigate the rôle of skin and of muscle in ganglionic responses. In the first series of experiments two embryos (Stage 25) were grafted together in parabiosis (Fig. 11). A region of skin from the left side of one component and from the right side of the other was excised and the embryos were then brought together with wounds apposed. This was done in order to bring about a considerable skin loss without a corresponding muscle loss (Detwiler, 1926b).
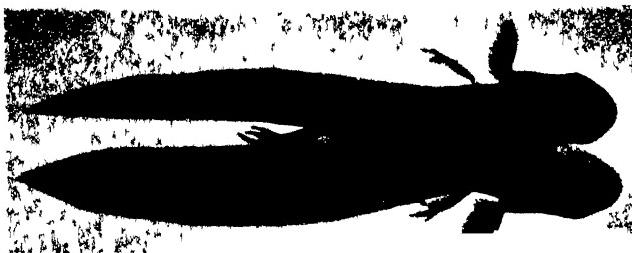
Fig 11. Dorsal view of parabiotic twin T 44 taken 44 days after fusion ×2 6.

These cases were sectioned subsequently and a quantitative determination of the skin loss, the muscle loss and the ganglionic cell loss through the region of fusion was determined. The figures thus secured were compared with those obtained from a study of the corresponding free sides of the two components in three sets of parabiotic twins. These data were brought together into three pairs of simultaneous equations which could be solved for consistent values of the coefficients. They showed that approximately 60 per cent. of the cellular reduction in the ganglia following fusion was due to skin loss and that approximately 40 per cent. was due to muscle loss. These figures were regarded as an index of the proportion of exteroceptive and proprioceptive neurones normally existing in the ganglia.

Conditions converse to the ones described, *i.e.* extensive muscle loss and only slight skin loss, were brought about by excising the lateral and ventral portions of the right sixth, seventh and eighth somites (Detwiler, 1927). A narrow medial wall of cells was left intact in order that any crest cells lying medial to this wall might not be disturbed or accidentally excised. As in the parabiotic twins, the embryos were studied with respect to the amount of skin loss, muscle loss and spinal ganglion cell loss in those nerves which supplied the defective area. The methods of obtaining the

quantitative data were described in a paper dealing with the parabiotic twins (Detwiler, 1926 b). These data were also brought together into simultaneous equations, the solution of which gave full support to the conclusions drawn from the results of the twin experiments, viz. that approximately 60 per cent. of the cellular reduction in the ganglia could be attributed to skin reduction and 40 per cent. to muscle loss.

In some recent experiments dealing with the transplantation of differentiated limbs, Carpenter and Carpenter (1932) estimated quantitatively the amount of both skin and muscle increase, and, using my figures for the relative proportions of skin-sensory and muscle-sensory elements in spinal ganglia, they calculated the expected hyperplasias in the ganglia supplying the grafted limb. Subsequent cell counts of the ganglion cells fell very close to the calculated hyperplasias.

The occurrence of these characteristic responses (hypoplasias and hyperplasias) of the developing ganglia to alteration of the peripheral territory naturally raised the question as to whether such responses can be elicited only when the peripheral changes are brought about in early embryonic life or whether they can be accomplished in later periods of development. This matter has been studied by Carpenter (1932, 1933) who grafted limbs at successively older stages from the early embryo (Stage 29) up to and beyond metamorphosis. Whereas in the early stages the limb bud is grafted before the peripheral nerves have developed, these experiments on larvae and on metamorphosed animals involve the grafting of fully differentiated limbs, as well as the interruption of fully formed nerves. Carpenter found that larval limbs, when grafted, acquire nervous connection with the host and that the nerves supplying the grafted limb undergo a hyperplasia similar in magnitude to that occurring when the limb buds are grafted before the outgrowth of peripheral nerves. When limbs are grafted after metamorphosis, the hyperplasia is not so extensive.

In a discussion of this matter Carpenter has drawn attention to the point that, whereas in embryonic life this ganglionic response might result from some bioelectric effect exerted by the rapidly growing limb (Herrick, 1925) such a factor could not explain the results in older larvae inasmuch as the limb has already passed through the phases of active growth and differentiation long before it is transplanted. Furthermore, the distance between ganglion and periphery is greatly increased by this time.

Since in Carpenter's experiments the ganglia were well differentiated at the time of the peripheral overloading, the question naturally arises as to the source of the additional neurones. Since the sheath cells have been shown by Harrison (1924a) not to contribute to the nerves, and since there is no evidence that differentiating and functioning neurones are capable of further proliferation, it is apparent that in the ganglia there exist cells of a "reserve or indifferent" nature which are capable of active proliferation. In this connection Carpenter draws attention to the fact that in the ganglia of older larvae and young metamorphosed animals, there exist centrally situated cells which are smaller than the mature ganglion cell, and have only a thin rim of cytoplasm around their nuclei. The possibility of these being the source of the additional neurones is considered theoretically by Carpenter.

Whereas it is not known yet what the factors are which are responsible for sensory hyperplasia in the ganglia, it is known that the nutrient level of the animal plays a rôle, for in recent experiments (Carpenter and Carpenter, 1932) it has been shown that in maximally fed animals the sensory hyperplasias are no greater than in those in which only moderate feeding has been given, but in "starved" animals the spinal ganglia which are subjected to overloading are able to respond only partially or not at all.

The above experiments show, therefore, that ganglionic hyperplasia is not restricted to embryonic life but that it may occur throughout larval existence and even after metamorphosis. Experiments designed to test how soon ganglionic responses to limb excision and limb grafting take place in the embryo have been studied in a preliminary way by Muschenheim. His results (unpublished) show that if this operation is made on embryos in the tail-bud stage, there is no detectable difference in the ganglia of the two sides up to the eleventh or twelfth days after operation, but from then on the effect of limb excision or transplantation becomes apparent by either hypoplasia or hyperplasia respectively. It is interesting to note that the time when this response in the ganglia is first observed corresponds closely to the period when the limbs begin to function.

VI. DEVELOPMENT OF PRIMARY MOTOR NEURONES FOLLOWING LIMB EXCISION AND TRANSPLANTATION.

Whereas the limb-grafting experiments have shown a marked reaction (hyperplasia) on the part of the afferent (primary sensory) neurones to peripheral overloading, no evidence as yet of a similar response on the part of the efferent (primary motor) neurones has been obtained either from a comparative study of the size of the motor roots or from a numerical comparison of the motor nerve cells in both halves of the spinal cord at the levels involved. Nor was there any measurable evidence of a hypoplastic development of the efferent fibres in the limb nerves (1920a, 1923a) as a result of the excision of the limb, though such nerves did suffer a reduction in size.

A study of size changes in the primary brachial motor neurones following limb excision in *Amblystoma* (Detwiler and Lewis, 1925) showed that bilateral excision causes a greater reduction in the size of the ventral horn cells than when only one limb is excised. In the latter case the average area of the median plane of section of the motor horn nuclei (planimeter estimates) shows a reduction of 8 per cent., whereas the volume reduction of the motor roots is 24 per cent. Following bilateral excision of the limb rudiments the average area of the median plane of section of the motor horn nuclei shows a reduction of 20 per cent., and the volume reduction in motor roots is 32 per cent. These results suggest that the size of the efferent neurones, in addition to being affected by the completeness of their functional connection with the peripheral field, is also dependent upon reflex connection with local commissural neurones of the opposite side of the cord. The results indicate that growth and function in groups of neurones may affect growth processes in

others regardless of whether or not the latter be fully or only partially in connection with the peripheral field.

From the observed facts that proliferation in the primary brachial motor neurones is apparently unaffected by the excision of the limb rudiment, and that efferent neurones from atypical regions of the spinal cord fail to undergo hyper-plastic development when in connection with a terminally increased musculature (transplanted limb), it is obvious that the extent to which brachial efferent neurones shall develop is not primarily under the control of the peripheral musculature. These findings are therefore not in accord with those of Shorey (1909), of Dürken (1911), and more recently of May (1930). May grafted a piece of spinal cord in the neigh-bourhood of the hind-limb rudiment in frog embryos (*Discoglossus pictus*). When such grafts did not supply the hind-limb, the cord lacked ventral horns, and the ortho-topic host cord was normal. In cases where the grafted cord "inhibited" the development of the hind-limb, there was no lumbo-sacral plexus on that side, in which case the host's spinal cord suffered a reduction in both the white and the grey matter. Also in several cases the implanted cord developed a large nerve which usurped the function of the normal lumbo-sacral plexus, and here again the normal cord underwent a reduction of the anterior horn cells on the side of operation. This, in some cases, was as great as 15 per cent. (*vide* May, *op. cit.* Table 4, p. 374). These responses differ from those obtained upon the fore-limb of *Amblystoma* embryos. In no cases in *Amblystoma* larvae, ranging from 30 to 60 days after limb excision, could any numerical difference be found on the two sides of the brachial cord, although the operated side may be somewhat reduced in size, owing to the great reduction in function. Likewise, when one, two or even three limbs are grafted to a heterotopic position and are innervated chiefly by the sixth and seventh nerves, the ventral motor areas of these nerves do not show any numerical increase in response to the added musculature. Regulatory compensation for the added peripheral territory is accomplished by increased division of the peripheral axons. This same observation has been made by Weiss (1931b), who studied the nerve supply to supernumerary limbs of the frog.

Not only does the cord fail to suffer any cellular reduction in the absence of the fore-limb in *Amblystoma*, but even in the complete absence of several myotomes (Fig. 12), no measurable cell reduction could be obtained on the side of the cord facing the denuded area (Detwiler, 1929a). In several cases in which the right sixth and seventh myotomes were entirely absent, cell counts were made in the left and right halves of the cord through this region. There resulted a 1:1 ratio. Moreover, comparisons of the weights of the grey and the white substance of the two sides showed only a very slight difference (Detwiler, *op. cit.*, Tables 1 and 2) which is to be expected, since on the right side there is complete absence of function. In the absence of muscle the motor roots on that side, in several instances, were almost as large as on the normal left side and no asymmetry in the gross morphology of the cord could be detected.(1929a, Figs. 3–18). The growth of motor roots in the com-plete absence of somites was also observed by Lehmann (1927). These results, therefore, supplement those obtained from limb excision and transplantation and

they offer additional support to the view stated previously that the proliferation of motor cells in the spinal cord of *Amblystoma* is not primarily influenced by the presence of functioning muscle, and that we must look therefore within the central

Fig. 12. Photomicrograph showing unilateral absence of the third, fourth and fifth myotomes. ×40.

nervous system itself for forces which appear to be chiefly responsible for cellular proliferation therein. May's experiments cannot be interpreted in line with my own findings and the whole matter needs further investigation.

VII. EXPERIMENTS UPON THE SEGMENTATION OF SPINAL NERVES.

Whereas there appears to be no question regarding the existence of a developmental, functional relationship between skin area, muscle mass and ganglionic cell proliferation, evidence has been produced also which shows a direct morphogenetic influence of the somites upon the ganglia. Working upon the urodele *Pleurodeles*, Lehmann (1927) excised the somites in embryos shortly after the closing of the neural folds, and also grafted a region of the spinal cord lateral to the host's somites. From his findings Lehmann drew essentially the following conclusions: (1) that in the complete absence of the myotomes and the sclerotomes, differentiated spinal ganglion cells are either entirely absent or are extremely sparse; (2) "that the segmental arrangement of the spinal ganglia occurs only when there is a degree of normal arrangement of the mesodermal structures, and that almost any disturbances in the mesoderm are accompanied by an abnormal development of the ganglion cells"; (3) that the normal location of the sensory and motor roots is subservient to a normal arrangement of the mesoderm, and that disturbances in the latter bring

about alterations in the location of the outgrowing fibre bundles; (4) that it is the presence of the mesial surface of the somites which brings about segmentation and differentiation of the ganglia. The lateral surface of the somite lacks this formative quality.

In an earlier paper in which partial myotomectomy had been intentionally performed, a number of cases were obtained in which there was a complete lack of several somites, yet definite segmented spinal ganglia were found (Detwiler, 1929 a, Figs. 19–22). In these instances, however, some cartilage was present lateral to the ganglia, which according to Lehmann is sufficient to stimulate ganglion development and differentiation.

Recently (Detwiler, 1932 b) this whole matter was investigated upon *Amblystoma* under a variety of experimental conditions and the results bear out in part Lehmann's findings upon *Pleurodeles* in showing that the segmentation of the mesodermal structures (myotomes and sclerotomes) determines the normal segmental arrangement of the spinal ganglia. This is apparent not only from cases where the mesoderm has been removed, but also from cases in which a piece of cord (after being deprived of surrounding mesoderm) has been grafted to strange positions. Under both conditions the ganglia which do develop show an irregular distribution, are frequently incompletely segmented, and are usually very defective. On the other hand, a number of cases have been found in my experiments in which discrete ganglia have been present in the total absence of adjacent mesoderm—a condition which, according to Lehmann, does not exist in *Pleurodeles*. In this connection Lehmann (1927, p. 110) says: "No or little differentiation of ganglion cells takes place in site of a normal cord in the case of (*a*) complete lack of skeleton and somites, (*b*) a grafted medullary tube adjacent to other parts of the somite than its median surface."

When the axial mesoderm (before or after segmentation) is removed from a donor embryo and segments of its spinal cord are grafted lateral to the somites in a host embryo, irregular ganglia may develop between the donor cord and the lateral boundaries of the host's somites. These ganglia have been found to undergo considerable differentiation in the possession of dorsal roots. That the lateral surface of the somites inhibits differentiation of ganglion cells, as asserted by Lehmann for *Pleurodeles*, is not substantiated in the experiments upon *Amblystoma*. Bringing together two spinal cords which have previously been divested of mesoderm (segmented or unsegmented) does not suppress ganglion development and cell differentiation between the adjacent surfaces (Fig. 13). Under such conditions the normal numbers of ganglia are reduced, they are usually very abnormal in shape and size, and may be incompletely segmented, but ganglia can and do develop and differentiate.

Inasmuch as differentiation of ganglion cells cannot be totally suppressed by the removal of adjacent somites or presomite mesoderm, one is justified in regarding the crest cells as possessing a certain amount of self-differentiating capacity in the same sense that Harrison (1910a) found the embryonic neuroblasts to differentiate *in vitro*.

Lehmann's view that segmentation in the nervous system is not intrinsic, but is subservient to primary mesodermal metamerism, has been supported further by recent experiments (Detwiler, 1933). The somites lying above the right anterior limb rudiment (somites 2–5) were excised in order to study the growth of brachial nerves without the assistance of the brachial somites. The results showed that whereas brachial nerves were present, their normal segmental arrangement was upset. Typically the ganglia failed to undergo normal segmentation, resulting thereby in several large elongated ganglionic masses (Fig. 14a), possessing a variable number of both sensory and motor roots, which in some instances could not be homologised with the roots of the opposite side. In some cases there was a definite reduction in the number of peripheral nerves. Lehmann in his experiments showed also

Fig. 13. Photomicrograph showing spinal ganglion which developed between the host cord (left) and the grafted cord (right) in the absence of adjacent muscle and cartilage × 52

that in the complete absence of myotomes the formation of motor fibres never seems to be suppressed entirely.

The atypical arrangement of the spinal nerves in this series of experiments, the failure of complete and orderly segmentation of the ganglia, and the occasional complete loss of a spinal nerve, all support the view stated above that typical segmentation of the nerves is secondary to normal mesodermal metamerism.

Still more crucial evidence to show the rôle of the mesoderm upon the segmentation of the ganglia has been derived from experiments in which the three brachial somites (3, 4, 5) were removed and a group of four somites from a more caudal position (somites 7, 8, 9, 10) from another embryo were grafted into the wound. In this way an additional somite was interpolated above the limb rudiment. Most of these cases showed the presence of an additional ganglion and spinal nerve, which corresponded exactly with the additional somite (Fig. 14b). In another group of experiments an incision was made between the spinal cord and the somites. Both cord and somite surfaces were thoroughly cleaned in order that any neural crest cells

which might be present could be eliminated. All cases subsequently examined have revealed the presence of a normal number of spinal ganglia with typical arrangement. Hence it is apparent that either the crest cells are not yet present when the operation is done, or, if they are, they regenerate and segment according to the original somite boundaries. Consequently, when four somites occupy the place of three, the four


Fig. 14. Reconstructions of spinal cord, ganglia, nerve roots and myotomes in *Amblystoma* larvae (× 25). Sensory roots are in solid, motor roots in broken lines. (*a*) case ME–9, 50 days after operation. The second, third and fourth right somites were excised in Stage 27. (*b*) case TrSO–37, 47 days after operation. The third, fourth and fifth right somites were replaced by the right seventh, eighth, ninth and tenth from another embryo. Note the presence of a supernumerary ganglion associated with the additional myotome (3 A).

ganglia which develop are determined by the somites themselves, otherwise a lack of correspondence between the number of ganglia and somites would be encountered.

The reduction in the number of ganglia and spinal nerves following somite removal, and especially an increase following the interpolation of an additional somite, constitute strong support of Lehmann's view that the normal segmentation of spinal ganglia and nerves is determined by the mesodermal metamerism. There is no support of the generally held view that nervous metamerism is intrinsic, at least in the spinal cords of the forms which we have investigated.

VIII. CELLULAR PROLIFERATION WITHIN THE SPINAL CORD

It was pointed out above that, in *Amblystoma*, the sixth, seventh and eighth spinal segments, when in nervous connection with a grafted limb, failed to show any evidence of an increased cellular development in response to the added peripheral musculature, in spite of the fact that marked hyperplasia of the primary afferent neurones ensued. It was suggested that if these extra-limb segments of the cord, which are capable of producing but limited movements in transplanted limbs, could be substituted for the brachial region of the cord, it would present a condition whereby it would not only be possible to study more favourably the ability of the extra-limb segment to execute normal limb movements when in connection with
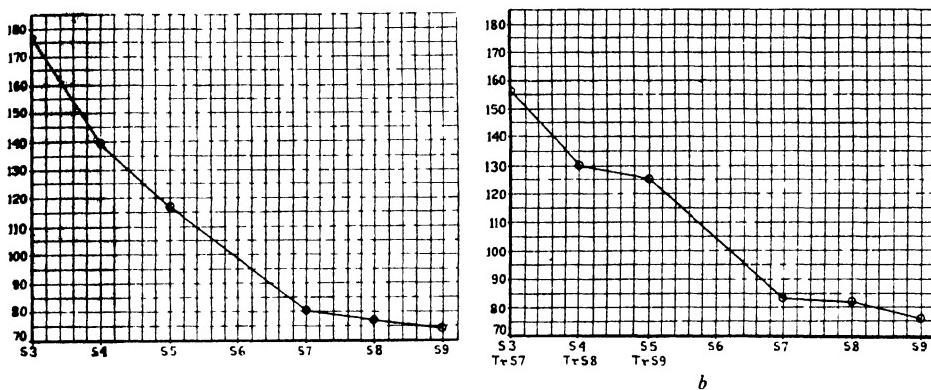

Fig. 15. Graph showing the extent of cellular proliferation within the right half of the spinal cord in *Amblystoma* larvae approximately 50 days of age Abscissae-designate the respective spinal segments (S 3–S 9); ordinates represent the mean number of cells per section as counted in twenty consecutive transverse sections through each nerve level The third, fourth and fifth segments are connected normally with the limb (*a*) normal larva (*b*) case TRSC–137, in which the normal limb segments (S 3, S 4, S 5) were excised in Stage 25–27 and replaced by the seventh, eighth and ninth (TrS 7, TrS 8, TrS 9) from another embryo

the proper central pathways, but the substituted portion of the cord would also be subjected to all the stimuli which normally produce the increased proliferation of nerve cells in the brachial region of the cord.

Consequently the brachial region (third, fourth and fifth segments) of the cord was excised in embryos ranging in development from Stages 23 to 28, and the seventh, eighth and ninth segments from another embryo were grafted into the excavated region (Detwiler, 1923 *a*). Larvae with these composite cords were found to be capable of normal swimming movements and in 50 per cent. of the cases the limbs functioned normally in spite of the fact that the brachial plexus was built up from strange segments of the spinal cord.

A microscopic study of the cords showed that the grafted segments in their new positions underwent an increased cellular proliferation so as to approximate to the number which normally characterises the brachial region (Fig. 15). Not only was

there an increased cellular production in the grafted segments, but they also underwent an increase in size and volume so as to approximate to the normal brachial segments (Detwiler, *op. cit.*, Table 8). Since extirpation of the anterior limb was found to have no effect upon cellular proliferation in the brachial region of the cord, the marked hyperplasia observed in the grafted unit appears to have no primary relation to the appendicular musculature, but must be occasioned by influences residing within the central nervous system. This conclusion received further support from a series of experiments in which the seventh, eighth and ninth segments, when grafted into the brachial region, underwent a similar hyperplasia in the complete absence of the limb (Detwiler, 1924*b*).

In a theoretical discussion of these results it was suggested that the increased cellular proliferation in the transplanted segments (7, 8, 9) might be the result of their being under the influence of a greater number of central longitudinal pathways descending from higher levels, such as the fasciculus longitudinalis medialis and the tractus bulbospinalis (Herrick, 1914). This view-point seemed further justified as a result of experimental end-for-end reversal of the brachial region of the cord (segments 3, 4, 5) which resulted in the development of a normal pattern of the spinal cord and peripheral nerves from the inverted segments (Detwiler, 1923*b*). Consequently these experiments were discussed in relation to Bok's (1915) "Stimulogenous Fibrillation" concept which has been embodied in Kappers' (1917, 1921) theory of neurobiotaxis. Attention was also drawn to the point that the observed cellular increases might be referred partly or entirely to the influence of axial physiological gradients as suggested in the work of Child (1921) and his students.

In referring to the cellular hyperplasias which were observed in the transplanted spinal segments, Coghill (1924) suggested that injury inflicted during the grafting may play some part in the extensive proliferation observed. He called attention to Hooker's (1915) observation on healing processes in transected spinal cords of *Amblystoma* and *Rana* in which injuries to the cord were found to produce proliferation by mitosis of the mantle layer. It was pointed out some time ago (1925*d*) that injury inflicted during the grafting probably plays no significant rôle in the observed hyperplasias. Recently (Detwiler and Maclean, 1932) an exhaustive study of this problem was made, by doubly transecting the cord at various levels, and by excising and replanting various groups of spinal segments which had been employed previously in various grafting experiments. The spinal cords studied at 8, 12, 16, 20 and 25 days after operation were compared with those of control larvae of similar age and length. No evidence of localized cellular increases resulting from injury could be obtained at any of the stages studied. In all cases, the curves of cellular proliferation at the various critical nerve levels in the experimental and the control cords were essentially alike (*op. cit.*, Table I, Figs. 8–11, 15–30). Only those larvae were studied in which the wound healed by first intention. Hence these cases cannot be compared with those described by Hooker (1915, 1923, 1925) in which there occurred a gap between the cut ends of transected cords, and in which restitution of the missing segment was accomplished in part by excessive proliferation of cells from the ependymal and mantle layers. The failure to find any evidence

of excessive cellular proliferation at any stage after 8 days in cords (thirty in number) transected and replanted at five different levels from the medulla to the ninth spinal segment, offers sufficient evidence, we believe, for concluding that when cellular hyperplasias result from the interchange of various spinal segments between different levels, the response can in no way be attributed to any stimulating effect of injury during the grafting.

In order to test the possible stimulative effects of developing bulbo-spinal fibres (Herrick, 1914) upon cellular proliferation in the cord, experiments were carried out in which an additional medulla was grafted just caudal to the normal (Detwiler, 1925*b*). The first five spinal segments were excised from the host embryo and replaced by a unit of central nervous system including the caudal end of the medulla and the first two segments of the cord. Under the new conditions the first three segments are replaced by the extraneous medulla and the fourth and fifth segments are replaced by the grafted first and second. The results showed a more extensive proliferation of cells in the spinal cord just caudal to the grafted medulla than occurs under normal conditions (Detwiler, *op. cit.*, Figs. 3 and 4). The cellular increase was most marked in the transplanted first and second segments and became less marked in successive segments.

These cellular increases were looked upon provisionally as resulting from the added stimulative effects brought about by the augmentation in the descending bulbar tracts arising in the extraneous cord and passing ventrally into the cord. In discussing the question of the action of gradients in the process of growth, Herrick (1925) has looked upon this cellular increase in the cord as being the direct result of the introduction of a new centre of dominance into an atypical place.

The reciprocal of this experiment was done by Nicholas (1928, 1929, 1930) who permanently blocked off the medulla from the cord by interpolating a limb or pronephros between the two. Nicholas reports that the cord under such conditions of development suffered a cellular hypoplasia of approximately 40 per cent. In addition, he shows the cord to be cylindrical in shape rather than possessing its usual tapering form—a condition which has not been obtained by Severinghaus (1930) and myself in isolated cords. Since Nicholas found that permanent isolation of the cord from the mid-brain had no effects upon cord development, he concluded also that the hypoplastic and atrophic cord development following isolation from the medulla is due to a lack of stimulation normally effected through descending bulbo-spinal fibres. It is interesting to note in this connection that Williams (1931), who isolated the lumbo-sacral region of the developing chick cord from the remainder of the central nervous system, also obtained a hypoplastic development similar in magnitude to that obtained by Nicholas.

In some recent experiments by the writer (unpublished) the medulla was excised and replaced by a unit of spinal cord comprising the seventh, eighth and ninth spinal segments. This embryo was then parabiosed to a normal embryo which served as a nurse. In several cases studied in a preliminary way, in which both nurse and experimental animal were of approximately the same size when fixed, the cord of the experimental animal was found to be considerably smaller than the cord of the

nurse larva, although it still retained its typical taper rather than assuming a cylindrical shape as found by Nicholas in permanently isolated cords.

The general interpretation which the writer made as the result of the original medulla transplantation experiments became subject to certain modifications in consequence of further experiments upon the cord (Detwiler, 1925 c). These involved the substitution of the first three spinal segments for the fourth, fifth and sixth, or for the third, fourth and fifth (brachial segments). Under these conditions the grafted first and second segments of the cord occupied the positions of the fourth and fifth respectively—a position occupied by them in the former medulla experiments (*vide supra*). The difference lies in the fact that in one case the transplanted segments are preceded by an extraneous medulla, whereas in the other, they are
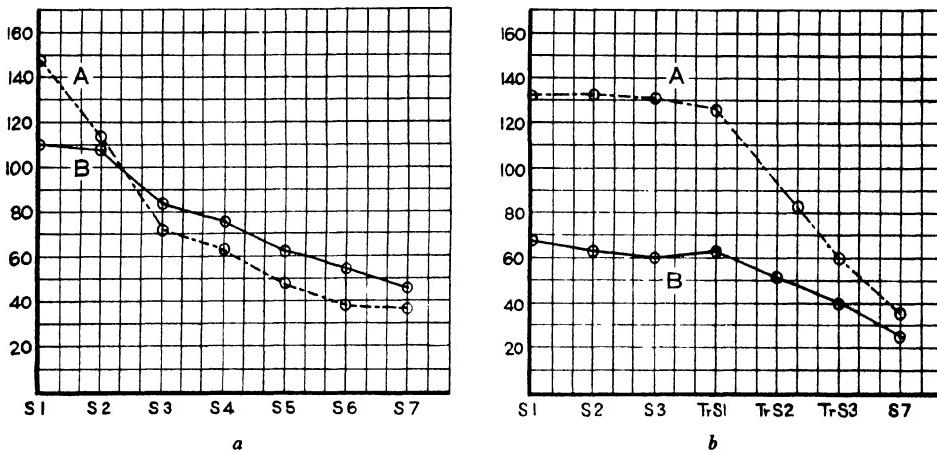

Fig. 16. Graph showing extent of cellular proliferation throughout the dorsal (sensory) region (*A*) and the ventral (motor) region (*B*) of the spinal cord in *Amblystoma* larvae. Abscissae, respective spinal nerve levels; ordinates, the mean number of cells per section as counted in ten alternate sections through each nerve level. (*a*) normal larva 50 days old. (*b*) case TrSC–23, 33 days old, in which the fourth, fifth and sixth embryonic spinal segments were replaced by the first, second and third from another embryo.

preceded by the normal anterior three segments of the cord. These results showed that the anterior end of the cord (first and second segments) when moved caudally in the embryo exhibited only slight decreases in cellular proliferation. The grafted segments in the more caudal position were found to approach their typical size and cellular content. The cellular development in the grafted segments was found to take place more extensively in the dorsal than in the ventral regions (Fig. 16 b). Moreover, cellular proliferation in the intact spinal segments cephalad to the transplanted ones was found to be markedly increased, particularly throughout the dorsal (sensory) regions, and the shape of these anterior intact segments simulated that of the caudal end of a normal medulla. This striking difference between proliferation throughout the dorsal and the ventral regions of the grafted segments as compared with the normal, suggests that in these segments capacity for proliferation is

much higher in the dorsal than in the ventral region of the cord. In other words the dorsal region has been shown experimentally to retain its embryonic character for a longer period than the ventral (motor). This fact was pointed out also by Coghill in his exhaustive studies upon proliferation and differentiation of cells within the spinal cord of *Amblystoma*. The cellular hyperplasia throughout the dorsal region of the intact anterior segments of the cord under the experimental conditions points to a stimulative influence exerted upon these regions by the grafted segments lying caudal to them. This influence was found to be effected throughout the dorsal regions only.

It would appear from the character of these results that there exists in the dorsal region of the anterior segments of the cord, particularly in the first and second, sensory centres inherently of high capacity for proliferation and that a strong influence passes out from these centres, particularly in a cephalad direction. The question has been raised as to whether or not this influence may be associated with the extent to which ascending tracts arise in this region, for this region is one through which strong sensory pathways (spino-bulbar) are developed cephalad into the brain.

If these results can be viewed from the standpoint of the effects brought about through the influence of growing axons, it would seem that there are two main influences affecting the final proliferation in the anterior segments of the cord. These would show a correspondence with the direction in which the principal pathways are growing. When the grafted anterior segments are preceded by an extra medulla, cellular proliferation in them is increased, but the relations between dorsal and ventral proliferation are essentially typical; but when the anterior segments are grafted to the same levels, but with the first three segments intact, proliferation in the dorsal regions of the grafted segments is disproportionately high. The same is true for the intact spinal segments preceding them (Fig. 16).

Although numerous other experiments by the author involving interchange of spinal segments could be described (Detwiler, 1928*b*, 1930*b*) space will not permit a discussion of the results. Recently Maclean (1932) has carried out extensive experiments involving the substitution of more anteriorly lying segments for more caudal ones and has found that whereas such segments are always reduced in size as compared with the region from which they are taken, the number of cells in the grafted segments is greater, slightly less than, or equal to that typical of the normal location, but is never reduced as low as that characteristic of their new location. If the segments are reversed before grafting, then they always develop more than their normal number of cells. Her results, therefore, are not the reciprocal of mine in which more caudally lying segments when grafted into a more cephalic level, undergo hyperplasia practically up to that typical of the new location.

Although numerous agencies are at work in the embryological development of the nervous system, there is considerable evidence to show that nerve fibres growing into developing regions of the nervous system stimulate cellular hyperplasia, and that the prevention of such ingrowth results in hypoplasia.

Burr (1916*a*), who removed the nasal placode in embryos of *Amblystoma*

punctatum, found that the corresponding cerebral hemisphere failed to complete its development. He showed also (1916*b*) that regeneration of the hemisphere failed in the absence of the olfactory placode. Whereas in his earlier work he stressed the matter of functional activity of the end-organ as the provocative element, he later (1920) concluded that some stimulus associated with the *ingrowth* of the axons rather than the function of the end-organ influences cellular development in the hemisphere. In this connection Burr performed two series of experiments. In the first, the cerebral hemisphere with the adjacent olfactory placode was grafted to a region just caudal to the anterior limb region and was buried beneath the skin. In the second series, the same structures were so grafted that the olfactory epithelium was exposed to the exterior as under normal conditions. He found that the telencephalon was as completely organised in one type of experiment as in the other, thus suggesting that function had no influence on cellular production. From his two types of experiment he concluded that the ingrowth of the peripheral axons into the wall of the hemisphere and not the functional activity of the end-organ was the important agent influencing cellular production in the hemisphere. Burr's experiments, although interesting and instructive, are not entirely crucial since one cannot assume that because the grafted nasal epithelium is exposed to the external world (second type of experiment) it is functional in the accepted sense. This lacks experimental proof. Furthermore, it is important to know how far proliferation in the grafted hemisphere would have progressed in the entire absence of the nasal placode.

The part played by fibre ingrowth in cellular production receives more substantial support from Burr's (1923, 1930) later experiments in which he grafted an accessory placode adjacent to the normal and found that the augmentation of olfactory fibres thus entering the hemisphere brought about a cellular hyperplasia in the olfactory portion of the latter, or when an accessory nerve entered the diencephalon and brought about hyperplasia therein. He found further that when a placode from the large tiger salamander (*A. tigrinum*) was substituted for the placode of the small spotted salamander (*A. punctatum*) there occurred a cellular hyperplasia in the olfactory portion of the hemisphere resulting from an additional number of fibres growing from the larger sense organ.

In some experiments involving eye grafting upon *Amblystoma* (May and Detwiler, 1925) we found that eyes occupying the position of the otic vesicle developed an optic nerve which penetrated the medulla, the IX–X ganglionic complex as well as the V–VII. All of these centres which were penetrated by the optic nerve underwent hyperplastic development. May (1927) later reported the same results upon anuran embryos.

Pasquini (1927) grafted accessory optic cups adjacent to the normal in *Pleurodeles* and found that the optic nerves joined, and, where the enlarged nerve penetrated optic centres, hyperplasia ensued. This same experiment upon embryos of *Rana fusca* (Detwiler, 1929*c*) gave negative results in that the optic nerve of the grafted component never joined the normal nerve, but always frayed out in the loose tissue surrounding the bulb. Harrison (1929*a*, 1929*b*) and Twitty (1932) reported hyperplasia of the optic centres under the influence of the increased optic fibre ingrowth

resulting from the differentiating optic cups of *Amblystoma tigrinum* which had been substituted for those of *Amblystoma punctatum*.

The exact manner in which axons growing into a region bring about increased cellular development is difficult of analysis. At present there seems to be no valid objection to interpreting the results in line with Cajal's neurotropism theory. Coghill (1924) has discussed this matter and brings out the fact that nerve cells grow while they function and suggests that the power of one neurone to activate growth processes in another has origin in the growth phase rather than in the conduction phase of its metabolism. In this connection he cites the experiments of Harrison (1904 *b*) in which he narcotised frog embryos in a 0·02–0·03 per cent. solution of chloretone during the period when the early nervous pattern is being laid down and found that, upon their removal 5 days later, they were able to complete normal swimming movements in the course of about 5 min. Here the total swimming pattern is laid down through successive developmental stages without ever having functioned. Certainly this type of experiment argues for an influence other than that brought about through nervous conduction. More recent experiments (Matthews and Detwiler, 1926) with narcosis of *Amblystoma* embryos corroborated Harrison's results, and showed that with longer narcosis the duration of the recovery period was increased—although animals left under constant anaesthesia for 13 days in a 1:3000 solution were able to recover.

Although Coghill (1924) admits that in later stages of growth the central nervous system may be influenced by ingrowing peripheral nerves, he also points out that the resolution of indifferent cells into neuroblasts in the very beginnings of the real neuronic system must be correlated with more elementary processes, since in various parts of the nervous system (*e.g.* rhombencephalon and spinal cord) the differentiation of neuroblasts has gone on at a comparatively rapid rate without any possibility of activation by ingrowing nerve roots since the root fibres of the ganglia do not grow in until a later period.

IX. HETEROTOPIC SPINAL CORD GRAFTS.

Although the experiments involving the interchange of spinal cord segments and the transplantation of the medulla have suggested that projection fibres invading the cord from higher levels may be responsible, at least in part, for the eventual degree of cellular proliferation which various segments undergo, recent evidence has been produced which indicates that the growth and connection of projection fibres may also actually inhibit cell proliferation by bringing about differentiation. This evidence has been suggested in the experiment by Severinghaus (1930) who grafted heterotopically units of cord into the lateral somitic region. The third, fourth and fifth segments with adjacent somites were grafted (with normal and reversed orientation) lateral to the third, fourth and fifth somites of the host. The sixth, seventh and eighth segments were likewise grafted under similar conditions lateral to the corresponding segments of the host. These experiments were done to study the effects of growth and proliferation of cells under conditions of total isolation of

the segments from any stimulative influences which might normally arise from the central nervous system itself. The results were as striking as they were unexpected in showing that the grafted segments not only grew to a larger size than the homologous intact segments (Fig. 17), but they proliferated cells considerably beyond the normal limits. Whereas proliferation of cells in the isolated cord units was most excessive in the dorsal (sensory) regions, where increases of nearly 300 per cent. over the normal were noted, hyperplasia in the ventral (motor) regions was obtained also (Severinghaus, *op. cit.* Fig. 4). He discussed his results in relation to Coghill's (1924) studies upon localised regions of alternating waves of proliferation and
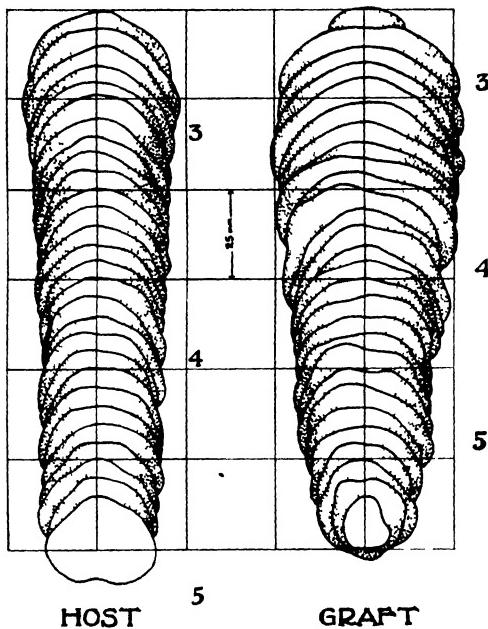

Fig 17 Reconstruction of the normal third, fourth and fifth spinal segments (host) and the grafted third, fourth and fifth segments (graft) in case 43 The donor cord along with the medial halves of the adjacent somites was grafted into the lateral somitic region of the host embryo. ×50 (After Severinghaus, 1930)

differentiation, and pointed out further the obvious inference that normally fibre invasion secondarily brings a halt upon cell division—since when the cells are removed from this influence, they continue to proliferate greatly in excess of the normal. He called attention to the possibility that certain projection fibres stimulate proliferation whereas others call forth differentiation. These two antagonistic influences acting upon the potential proliferative capacity at any given level of the cord would thus regulate the number of cells at various levels under normal conditions. Certain it is, from his results and from corroborative evidence by the writer, that the proliferative potential of a given region of the cord is much greater than what is normally expressed, and that there exists in the central nervous system some

powerful restraining influence upon cell division, otherwise cellular proliferation in the isolated cord units would not have run riot, so to speak. The results which Severinghaus obtained must be regarded as highly important to one who inquires into the dynamics of normal development of the central nervous system, and they incite further inquiry into the complicated processes of proliferation, growth and differentiation.

In some recent experiments by the writer (Detwiler, 1932*b*) a three-segment unit of supernumerary cord was grafted adjacent to the normal so that no mesoderm tissue existed between the two. Although this operation was performed to study the development of spinal ganglia in the absence of adjacent mesoderm, it was found in all cases that the heterotopic cord was smaller than the normal (Fig. 18), a condition in contrast to that obtained when they are grafted more laterally (somitic region) where they always become larger than normal. This size reduction ranged from 20 to 50 per cent. It was at first thought that this reduction might be due to restraining influences associated with projection tracts arising from the normal cord and invading the graft, since in all of the earlier cases examined the two cords were fused at some point. Cases were obtained later, however, in which the grafted cord with similar size reduction had no connection with the intact cord. This eliminated the possibility of any influence associated with projection fibre invasion. Since the cellular proliferation in these grafted cords is slightly higher than normal in spite of marked size reduction, it is clear that, whatever the nature of the inhibitory influence is, it affects general growth much more than it does cellular content. The reduced size could not be explained with reference to any restraint from mechanical influences of the surrounding tissue, for the grafted cords adjacent to the normal suffer a similar size reduction regardless of whether they lie within the same neural arches as the normal or whether they possess their own arches. The presence of much, little, or no surrounding cartilage seems to have no effect upon growth in size. A search for an influence on the part of the notochord also resulted negatively.

Fig. 18. Graphic reconstruction of normal cord (left) and grafted cord (right) in case SCMT–3 (57 days). The heterotopic cord was grafted adjacent to the normal in an embryo of Stage 28. The numbers indicate spinal nerve levels. ×25.

Since cellular proliferation is much greater in the more laterally placed cords than in those adjacent to the normal, it is clear that there is some sort of inhibitory influence which becomes weaker as the distance between normal and grafted cords increases. The fact that spinal cords grafted adjacent to the normal grow to smaller size without any connection of substance does not lessen the value of the suggestion by Severinghaus that certain invading projection fibres within the central nervous system may bring about differentiation and thus put an end to proliferation. These experiments merely indicate the presence of an additional restraining influence to

growth and proliferation which cannot be referable to actual projection fibre invasion, but rather to some condition associated with the nearness of two like structures.

This sort of developmental response indicates the presence of another obscure influence operating in nervous development. One can truly reiterate what was mentioned in the introduction, that, whereas our knowledge of these complicated interacting forces lies little beyond the theoretical stage, many interesting facts continue to accumulate and it is only through these that any sort of adequate concept of the dynamic forces at work can be appreciated.

Although it has been pointed out in this article that after the neural tube is closed there is only scant, if any, evidence for an influence of growing and functioning mesoderm upon cellular proliferation within the cord, one must not forget that in the early development of the neural plate abundant evidence has accumulated in the work of Spemann and Geinitz (1927), Lehmann (1926), Adelmann (1931), Goerttler (1925) and others demonstrating a definite rôle of the mesoderm upon morphogenesis in the neural plate. Unfortunately space does not permit a discussion of these and other investigations which constitute one of the most fascinating and highly important chapters in the field of experimental embryology.

X. HETEROPLASTIC SPINAL CORD GRAFTS.

During recent years students of correlative embryology have become much interested in problems of growth as studied by the method of heteroplastic grafting of organs between animals of two different species possessing different growth rates. The original experiments of Harrison (1924 b) in which he grafted limb rudiments between embryos of *Amblystoma punctatum* and *A. tigrinum* were so striking in their results as to stimulate many investigations in this field. Harrison found that limb buds grafted from the large *A. tigrinum* to the small *A. punctatum* remained very small for a time, but soon became accelerated in their growth, and the resultant limbs not only exceeded in size the limb of the host animal, but became even larger than the donor control limb. The reciprocal graft (*A. punctatum* to *A. tigrinum*) resulted in a limb which was smaller than that of either species. Other experiments (Harrison, 1929 a) gave similar results for the eye, ear and gills. In other words, the growth of the grafted organ became, for a considerable period, either accelerated or retarded beyond that of the donor control, according to which species was used as host. In order to account for the striking results, Harrison postulated provisionally a growth-regulating factor (R), other than nutritional ones, as present in the circulating medium of the organism and held to be stronger in *A. punctatum* than in *A. tigrinum*. An experimental search for this factor gave negative results.

Subsequent experiments by Twitty and Schwind (1928, 1931), using both limb and eye rudiments, showed that when the animals were fed maximally the voracious *tigrinum* larvae grew so much faster than the *punctatum* larvae that the difference in growth rate between normal and grafted organs became obliterated. Under such conditions of feeding the *tigrinum* rudiment upon the *punctatum* host grew with a velocity characteristic of the donor species rate.

These findings have been supported subsequently by Harrison (1929 *b*), Burns and Burns (1929) and Copenhaver (1930). The originally postulated regulating factor (R), which was first thought to be of the nature of a hormone, became identified, therefore, with the "nutrient level" of the organism (Harrison, 1929 *b*, pp. 45, 46). In the experiments, therefore, in which the *tigrinum* rudiments were grafted to the *punctatum* embryos, and grew at a greater rate than those of the donor control, it became apparent that the nutrient level of the donor animals was lowered by sub-maximal feeding as compared with the *punctatum* host animals.

Heteroplastic grafting of spinal cord segments was carried out by Wieman (1925, 1926) in connection with a study of the effects of a foreign source of innervation upon the development of the anterior limb. Whereas Wieman gave a general account of his experiments from this standpoint, he unfortunately gave no data upon the growth and size of the graft as compared with the corresponding portion of host and donor controls. Neither did he indicate whether the graft had any effect upon the intact portion of the host cord. These and still other matters, such as cellular proliferation and size regulation in the graft, seemed sufficiently important to investigate for comparison with the results obtained upon other organs. In the original experiments (Detwiler, 1931) the brachial region of the *tigrinum* cord was substituted for the corresponding portion of the *punctatum* cord. Larvae varying from 32 to 62 days after the operation were studied, and it was found that the *tigrinum* graft had regulated so as to correspond in size and cellular content with this portion of the *punctatum* cord. In the reciprocal experiment (*A. punctatum* to *A. tigrinum*) the graft developed in the *tigrinum* host as though it were a normal *tigrinum* brachial region.

In a later series of experiments (Detwiler, 1932 *a*) this same operation was carried out, but the larvae (host, donor control, and host control) were fed maximally and the growth of their spinal cords was studied in early stages ranging from 8 to 30 days after the operation. The results showed that the brachial region of *tigrinum* embryos, when substituted for the corresponding region in the *punctatum* embryos (Harrison's Stages 25–27), grew, in early stages, with a velocity which was greater than that exhibited by either the *tigrinum* donor control cord of the *punctatum* control cord. This was manifested by increase both in size and cellular content (Fig. 19). By approximately 30 days after the operation, the evidence for this early acceleration of growth had largely disappeared under the influence of regulatory processes in the growth of the cord as a whole. Since the graft in early stages grew with a velocity much in excess of the donor control correspondent under conditions providing for approximately maximal feeding on the part of both host and donor control larvae (Detwiler, *op. cit.*, Fig. 1), it is apparent that maximal feeding in this instance was not sufficient to obliterate the differential growth rate. In other words, the graft in early stages did not grow with a velocity characteristic of the donor control species. In spite of the accelerated growth of the graft in early stages (8–21 days) over both donor control and host control cords, regulatory processes play an important rôle in later growth. By 30–32 days after the operation the graft in the *punctatum* host is smaller than the donor control homologue regardless of the

nutrient level of the donor control animals, and from this period on it is regulated to the size normally attained by the corresponding region in a normal *punctatum* cord.

Evidence of accelerated growth under conditions of maximal feeding was obtained also from experiments in which the first three spinal segments from both
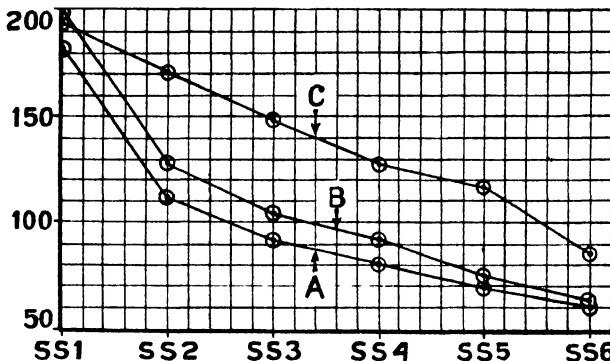

Fig. 19. Graph showing mean number of cells per section (ordinates) as counted in five alternate unilateral transverse sections of the spinal cord through each nerve level from 1 to 6 (SS 1–SS 6) in Exp. TrSCHB–5. *A, A. tigrinum* donor control cord; *B, A. punctatum* control cord; and *C, A. punctatum* host cord with the brachial regions (SS 3–SS 5) of *A. tigrinum* origin.
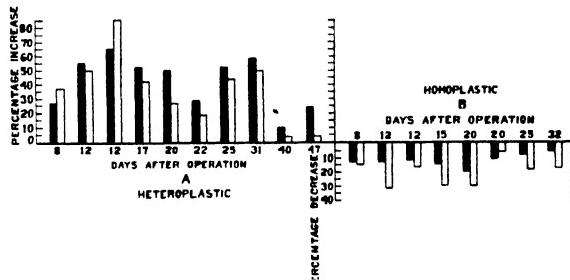

Fig. 20. Graph showing: *A*, increased growth of the grafted first, second and third spinal segments of *A. tigrinum* over the donor control segments when they occupy the brachial region (third, fourth and fifth segments) in embryos of *A. punctatum*; *B*, decreased growth of the grafted first three spinal segments of *A. punctatum* as compared with the donor control segments, when they are grafted homoplastically into the brachial region of the cord (segments 3, 4 and 5). The solid columns represent percentage cellular increases or decreases per section, and the unshaded columns the average size increases or decreases per section. The figures along the horizontal line designate time in days after operation. Each column represents an individual case.

tigrinum and *punctatum* donor embryos were substituted for the fourth, fifth and sixth segments of a *punctatum* host cord. In the homoplastic combinations the graft in all cases was slightly smaller than its donor control, whereas in the heteroplastic combination the graft in every case underwent marked accelerated growth over the *tigrinum* donor control. This acceleration was manifested by growth in size and in cellular proliferation (Fig. 20).

These results are not in agreement with those of Twitty and Schwind (1928, 1931) for eye and limb grafts. Regardless of maximal feeding, accelerated growth in the grafted unit of cord was obtained. It is apparent, therefore, that with the present evidence the increased velocity in growth of the graft over the donor control cannot be explained upon the basis of growth depending upon food intake. That factors other than the intrinsic growth rate are operative seems apparent, and it is also clear that this acceleration is very early regulated down so that in later stages (30 days and beyond) all evidence of it is lost.

Unless other influences associated with the nutrient level of the organism are discovered to account for this striking acceleration in the growth rate of the graft during early stages, it may yet be necessary to postulate some other accelerating influence associated with the host as a whole or with its central nervous system.

Just why heteroplastically grafted organs such as limb and eye rudiments should grow with a velocity characteristic of the donor species (under conditions of maximal feeding), and the spinal cord segments should show marked accelerated growth under similar nutrient conditions, is difficult of analysis and the whole problem undoubtedly awaits further experimentation in order that the matter of differential growth may be more fully understood.

It has not been possible in this communication to consider all of the experiments dealing with morphogenesis in the amphibian nervous system. The object has been rather to review only certain lines of experimentation as they bear upon this perplexing problem, and it is hoped that this résumé may be of assistance to those who are interested in the dynamics of developmental physiology of the nervous system.

XI. SUMMARY.

An account is given of certain experiments which bear upon the problems of morphogenesis in the central and peripheral nervous system. By means of grafting embryonic limbs and other rudiments it has been possible to alter the direction of growth in spinal nerves. These responses are discussed in relation to the probable nature of forces responsible for the general growth and connection of nerves.

Experiments involving limb and somite excision and grafting are described as they bear upon the development of primary somatic sensory and motor neurones, and upon the segmentation of spinal nerves. The results indicate a fundamental difference in the growth response of motor and sensory neurones to peripheral changes. They also support the theme that segmentation in the spinal cord is entirely subservient to mesodermic metamerism.

The autoplastic interchange of various embryonic spinal cord segments, as well as heterotopic grafting experiments, are described as they bear upon the problem of cellular proliferation and differentiation within the embryonic spinal cord. These results, in combination with those following limb and somite excision and grafting, indicate that forces within the central nervous system are chiefly responsible for proliferation therein, rather than growth influences from the periphery.

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PROGRAMME-EVOLUTION IN THE GRAPTOLITES

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(With Nine Text-figures.)

CONTENTS.

I. INTRODUCTION.

AMONG the more significant features of palaeontological literature during the present century may be noted, firstly, an accumulation of data concerning lineages and an insistence upon the view of a gradual and continuous process of change, and secondly, the prominence given to the conception, in some form or another, of the theory of Programme-Evolution. Both these concepts are opposed by the majority of zoologists, and neither is universally held among palaeontologists.

The question of continuity of change in a lineage or "species series" is to a large extent beyond the scope of the present paper. Most palaeontologists write as convinced opponents of mutational change, but some (*e.g.* Watson, 1929) are prepared to accept the multiple factor hypothesis of the geneticists and, recently, Swinnerton (1932) has put forward the view that the discrepancies between the

mutational and the transient theories can be explained largely as a result of the nature of the approach to evolution and of the methods of investigation. With reference to the graptolites, where the course of evolution is specially rapid and breaks in the lineages are not infrequent, the question cannot well be considered. Nor have we as yet much definite evidence as to the complexity or otherwise of the lineages, the relative simplicity of the skeletal parts possibly masking more complex evolutionary changes. It is probable that lineages (or in this sense, more properly, "gentes" or species groups) are more truly represented as a plexus of anastomosing lines (cf. Trueman, 1924) than by simple lines of descent, as it is bound to be where two or more characters are evolving independently and interfertility is not restricted to narrow limits.

For providing examples of programme-evolution, however, the graptolites are pre-eminent. The whole evolution of this group of animals was described in outline by Elles (1922) in terms of three or four distinct trends, and this article aims only at a slightly elaborated account and a general discussion of a few examples.

Where one or more characters are undergoing change in a definite direction, in any given lineage, we may substitute the term "seriation" for "filiation" and describe the series as orthogenetic. As Bather (1921) has pointed out, just as succession in time is not necessarily proof of true descent, so there may be seriation without determination and predetermination, but it is the very remarkable parallelism in the evolution of related lineages that has given rise to the theory of trends.

A trend may be defined as the tendency for a character to run through a similar developmental history independently in several lineages, and "because each character appears to carry out in its evolution a predetermined course, the process has been called Programme-Evolution" (Lang, 1923 b)[1]. It is suggested at the end of this paper that the term has been employed in several slightly different meanings, but none of the many writers on this subject has ever attempted to imply "anything of what is usually understood by teleology," which seems to be one of the main criticisms of their opponents. Most have put forward some tentative physiological explanation. Among biologists, Dendy (1912) has postulated a physiological basis for his momentum, while Duerden (1919) considers that the orthogenetic series in the ostrich may be "interpreted in terms of germinal senescence," and Vavilov (1922) concludes simply that "it seems as though Nature cannot differ indefinitely."

From the palaeontological side, the theory has been advocated in a series of papers by Lang, and has been taken up by many other writers. Instances of programme-evolution have been claimed to occur among practically every group of the invertebrates and it will suffice to mention here the graptolites (Elles, 1922, 1923), corals (see Lang, 1923 b), Polyzoa (Lang, 1917, 1921), brachiopods (Teichert, 1930; Fenton, 1931), echinoderms (Spencer, 1913), and Mollusca (Kitchin, 1912; Lang, 1919; Trueman, 1922). The evolutionary series described by Jones (1928) in *Plectambonites* and allied brachiopods, by Kiaer (1908) in *Pentamerus*, and by Rowe (1899) in *Micraster*, might well be cited as additional examples.

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[1] The actual name was proposed by Dr F. L. Kitchin.

II. GENERAL MORPHOLOGY OF THE GRAPTOLITES.

The graptolites[1] are a group of colonial organisms which originated, developed and died out in the Lower Palaeozoic era. Of their zoological affinities little is known with certainty, but it is possible that they are to be regarded as a group of the Coelenterata. Some authors have considered them to be related to the Polyzoa (Ulrich and Ruedemann, 1931) and others again (Wiman, 1895; Elles, 1922) hold that they are best considered as a separate group. Commonly their remains consist merely of carbonaceous films on the bedding planes of shales and mudstones, but in more favourable conditions of preservation, as, for instance, in those that occur in limestones and siliceous nodules, the chitinous periderm of the colony or rhabdosome is preserved in full relief and may be completely freed from the matrix by the use of appropriate acids.

The earliest stage of any graptolite rhabdosome is the conical first cell or sicula, which differs in many important respects from any of the cells subsequently developed. The apex of this cell is prolonged into a fine thread or nema, by means of which, it appears, both the sicula and, later, the entire rhabdosome was attached to floating seaweed[2]. In some diplograptids and monograptids, it seems that the nema itself underwent modification, with the development of a terminal vesicular body which is interpreted as a float, so that these graptolites were perhaps truly planktonic; and in many such species, a large number of separate rhabdosomes is seen to be grouped around a central vesicle.

A single bud is produced from the sicula, and by the subsequent division of this bud and its descendants the various types of branched rhabdosome are produced. Different modes of development of the proximal end characterising different genera have been described by Elles (1922) and the writer (1932) and possess an evolutionary significance.

When the history of the graptolites is traced from their origin in the highest Cambrian rocks (Tremadocian – Lower Ordovician of continental authors) to their final extinction in the Upper Silurian, certain definite trends can be recognised; that is to say, through all the diversity of form, development takes place in relatively few and clearly defined directions in the many different lineages. Within comparatively narrow limits, this evolution takes place at a comparable rate in all the different lines of descent, with the result that the whole aspect of the graptolite fauna changes as we pass upwards in time, and it is possible to recognise the major geological horizons by the stage of evolution reached by the fauna as a whole, irrespective of generic or specific identifications. On this basis, four graptolite faunas, dichograptid, leptograptid, diplograptid and monograptid, have been described (Elles, 1922) and within each of these, further subdivisions are possible on general evolutionary principles

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[1] We are considering here only the true graptolites or Graptoloidea; the dendroid graptolites differ in their structure, development and geological range, although clearly related to the Graptoloidea.

[2] This hypothesis (due originally to Lapworth and elaborated by him in 1897) accounts both for the remarkably wide geographical distribution of the graptolites and for their typical occurrence in black, *carbonaceous* shales.

before passing to the finer zonal subdivisions. At the same time, most if not all of the genera are polyphyletic and represent grades or stages of evolution rather than genetic relationship.

The number of characters which can be studied in the graptolites is relatively small, and the characters of the thecae have proved the most reliable index of affinity in tracing their evolutionary history.

It has been shown by Elles (1922) that the three principal trends of evolution which are followed by the graptolites are: firstly, reduction in the number of branches or stipes (stipe-reduction); secondly, change in direction of growth of the stipes relative to the suspensory nema, leading from pendent or horizontal to scandent forms; and thirdly, elaboration of thecal type. This evolution is in two respects remarkable, because one trend (that of stipe-reduction) is entirely a simplification process, and, again, the manner in which new structures (thecal types) are introduced is contrary to that in most other groups of animals. Thecal elaboration, particularly in the monograptids, is first seen at the *proximal* end of the rhabdosome and gradually spreads along its length. Thus it is the initial thecae which exhibit the first signs of incoming developments (see Fig. 1) and, during retrogressive changes, it is these thecae which retain longest their elaborated character. Recapitulation, as commonly understood, cannot occur here; only in the change in direction of growth trend, and in the delayed bud formation (*vide inf.*) is recapitulation to be recognised.

Fig. 1. *Monograptus argenteus* (Specimen 747, Sedgwick Museum), *M. gregarius* Zone, Skelgill Beds, Skelgill. × 5 approx. The species is an early member of the hooked series of thecal elaboration in the monograptids. The arrows, drawn at right angles to the hydrothecal apertures at various points along the stipe, indicate the degree to which the thecae are reflexed and illustrate the introduction of a new thecal type beginning at the proximal end of the rhabdosome (lower portion of the figure).

Trend i. Simplification in branching.

The true graptolites are probably descendants of the Dendroidea, and their earliest representatives are multiramous genera in which the stipes may number as many as sixty-four, produced by a regular process of dichotomy. The history of the Dichograptidae, to which family these genera belong, is very largely the story of the gradual reduction in number of the branches, and in general this reduction is in such a manner

as to produce at each stage a symmetrical rhabdosome. It is now recognised that nearly all the dichograptid genera represent simply these reduction stages and imply no close phyletic relation. Occasionally asymmetrical colonies are produced and in these cases the distinguishing feature lies, not in the actual number of branches but in the maximum degree of dichotomy attained in any part of the rhabdosome. In the general history of the succeeding dicranograptids, diplograptids and dimorphograptids, may be seen a progressive delay in the formation of the two buds from which are produced the independent linear series of cells which occur in *Dicranograptus* and the more primitive biserial rhabdosomes; and it has lately been suggested (Bulman, 1932) that this process is but a continuation of the stipe-reduction trend of the earlier dichograptids.

Regarding this process of stipe-reduction, however, as a trend essentially of the Dichograptidae, we find that it there operates independently in about ten parallel series among British species (Elles, 1922, table), while Ruedemann (1919) shows some fourteen such lines among the species of eastern North America. Doubtless many others will be revealed in the course of future work, and there are other districts where identical or parallel lineages are undoubtedly present, but have not yet been worked out in detail.

Concerning the origin of these trends, Ruedemann (1919) holds that the evolution, though "typically orthogenetic," is "the result of persistently acting exterior factors such as the advantages of equal distribution of food to all branches and the attainment of equilibrium of the floating rhabdosome by symmetric and uniform development of all branches"; and that there might be some such "orthodox" explanation was previously suggested by Elles (1923, p. 90): "This simplification in branching may, as suggested by Nicholson and Marr, be the impression of the struggle for an adequate food supply." Reasons for considering these explanations as perhaps only partially adequate to account for the origin of this trend are discussed on p. 331.

Trend ii. Change in direction of growth of the rhabdosome.

The ancestral genera possessed branches which were, assuming the rhabdosome to have hung suspended by the nema, either pendent or horizontal, and during the earlier stages of the stipe-reduction trend there was no change in respect of this direction of growth. Later, however, the horizontal forms exhibit a tendency towards the production of colonies in which the stipes were reclined and eventually erect (or scandent)[1]. An offshoot of the four-stiped *Tetragraptus* attains the scandent direction of growth in the genus *Phyllograptus*, but otherwise the change is one affecting only the two-stiped *Didymograptus* and its descendants, after or accompanied by various changes in the mode of development of the proximal end. The general direction of this trend is represented by the form-series *Didymograptus—*

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[1] Except that the horizontal *Clonograptus* is probably to be regarded as a derivative of the pendent *Dictyonema*, the pendent forms seem to have been unaffected by this trend and suffered extinction after the stipe-reduction trend had run its course, at an appreciably slower rate than in the horizontal forms.

Leptograptus—Dicellograptus—Dicranograptus—Diplograptus and its fullest expres-
sion is seen in the last-named genus, where the stipes grow up and enclose the nema,
and in the uniserial, scandent, *Monograptus*. In more advanced types of *Diplo-
graptus* and in *Monograptus* and its allies, both the stipe-reduction and the scandent·
direction of growth trends have reached their acme.

It has been suggested that the origin of this trend is to be seen in the measure of
protection afforded to the important but vulnerable nema attachment, although if it
is regarded as an example of Darwinian selection, it must be admitted that the
attainment of the end was a remarkably slow process, conferring little advantage in
its early stages, during which forms with an unprotected nema survived with con-
siderable success.

Trend iii. Elaboration of thecal type.

Under this heading have been brought together a number of changes affecting
the shape of the hydrothecae in different families of graptolites. Originally the
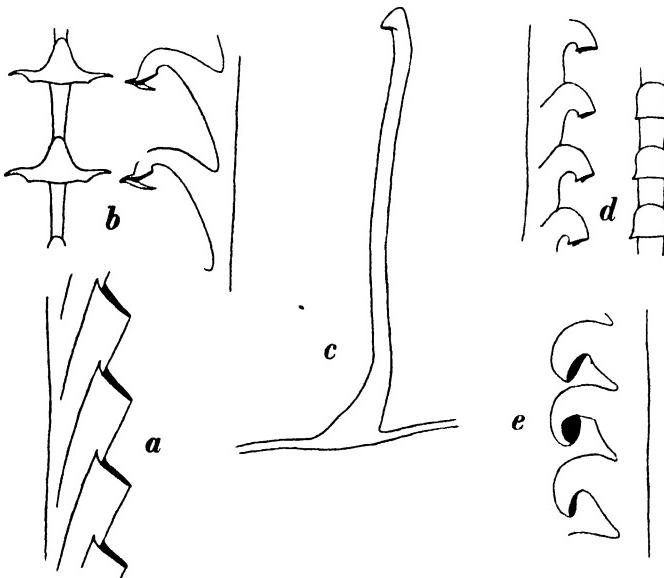
Fig. 2. Restorations showing various types of thecal elaboration in the monograptids. *a*, simple
thecae; *b*, triangular thecae, with apertural spines; *c*, isolate thecae (acme); *d*, hooked thecae (acme);
e, lobate thecae (acme). Figures *b–e* based on actual specimens; figure *a* diagrammatic.

thecae were simple, straight, overlapping tubes, and they remain of this type through-
out the Dichograptidae, with three or four exceptions among the later didymograp-
tids. These exceptions are of the same type as the modifications which occur in the
Leptograptus—Dicellograptus series and of which there are parallel developments in
the genera *Dicranograptus, Climacograptus* and in some *Diplograpti*. Here the straight
Dichograptus type of cell becomes progressively more and more sigmoidally curved

or even bent at a sharp angle, and in later stages the apertural region shows intro-version and sometimes introtortion (twisting on its axis). The youngest dicello-graptids of the uppermost Ordovician are again, like the late leptograptids, simple-celled forms; but whether this is a reversion to, or a persistence of, the more primi-tive type is not certainly known.

More important from our present standpoint are those thecal modifications which affect the monograptids; forms which, it has been noted, have reached the limits of development along the simplification of branching and scandent direction of growth trends. The thecal trends recognised here are those of isolation and lobation, the latter further divided into the lobate and the hooked series (see Fig. 2). These, as pointed out by Elles (1922), do not remain entirely distinct, for isolation is accompanied by a certain amount of lobation and *vice versa*, but one or other is dominant in any given line. The hooked line, represented for example in the series *M. cyphus—M. tumescens* (described in detail by Elles, 1923), shows regressive phases after reaching its acme or fullest expression in *M. priodon*; the lobate line terminates in forms like *M. lobiferus* and *M. crispus* without any return to simpler type; and the isolate trend, although it does not show any regression after its acme on the main line, has certain regressional offshoots.

While we have still only an imperfect understanding of the true phylogeny of the monograptids, it is perhaps not without value to take stock of the results that have already been obtained both in this country and abroad, and of interest to consider the direction in which these results are tending. In this connection, the so-called isolate line offers the most promising developments.

III. THE ISOLATE LINE OF THECAL ELABORATION.

The isolate development, culminating in such extreme forms as *Rastrites maximus*, has been recognised in the evolutionary studies of Eisel (1912) and Elles (1922, 1923). The latter authority sees the first evidences of the series in the species *Dimorphograptus decussatus* Elles and Wood, from which she derives *Monograptus raitzhai.iensis* (Eisel); and it was realised by Törnquist as long ago as 1907 that *M. raitzhainiensis* and *M. triangulatus* (Harkness) are succeeded by "a group of unquestionable *Monograpti* characterised by the fact that a number of the oldest thecae are linear and isolated, while the following ones are triangular and in contact with each other though without overlap. For all I know this group does not range up to the top of the *Rastrites* division" (Törnquist). For these forms, intermediate in structure between *Monograptus* and *Rastrites*, the generic name *Demirastrites* was proposed by Eisel. The evolution of the forms known as *Rastrites* is simply a question of a further development by which all the thecae of the rhabdosome become isolated and linear, accompanied by a remarkable attenuation of the stipe itself.

(1) *The* Rastrites maximus *series* (Figs. 4 and 5).

The probable terms of the series *Monograptus raitzhainiensis—Rastrites maximus* are shown, together with their zonal range as known at present, in Figs. 3 and 4.

In *M. raitzhainiensis*, the first theca is adpressed to the sicula for the greater
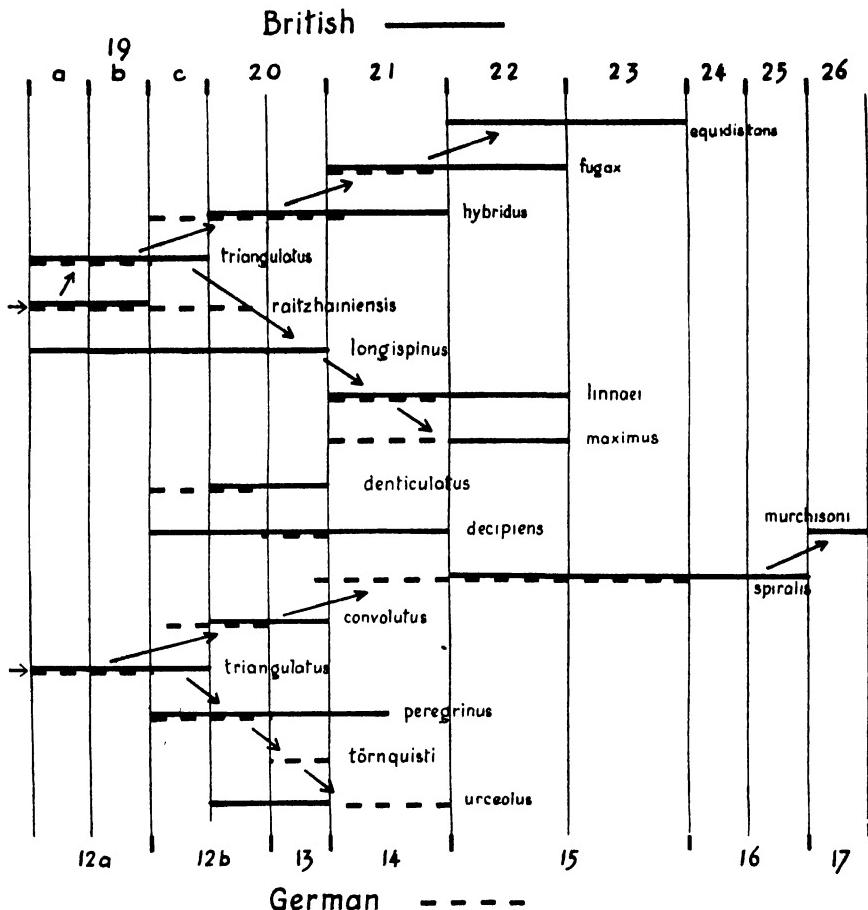

Fig. 3. Some morphogenetic series in the isolate type of thecal elaboration in *Monograptus*. The range of species in Britain (on the British zonal scale, zones 19a–26, Elles and Wood, 1902–18) shown by continuous lines; the range in Germany (on the German zonal scale, zones 12a–17, Eisel, 1912) shown by broken lines. The range of species in Britain is taken from Elles and Wood, 1902–18 (Table I) and the range in Germany is that given by Eisel (1912).

part of its length; thecae 2–8 are tubular and isolate, with the aperture situated at the end of the tube; and beyond this region the thecae broaden in the adnate portion and finally become completely triangular. There is little doubt that this species must have been preceded by a form of *Monograptus*, as yet unrecognised, in which all the thecae were triangular in shape. These triangular thecae imply a certain amount of
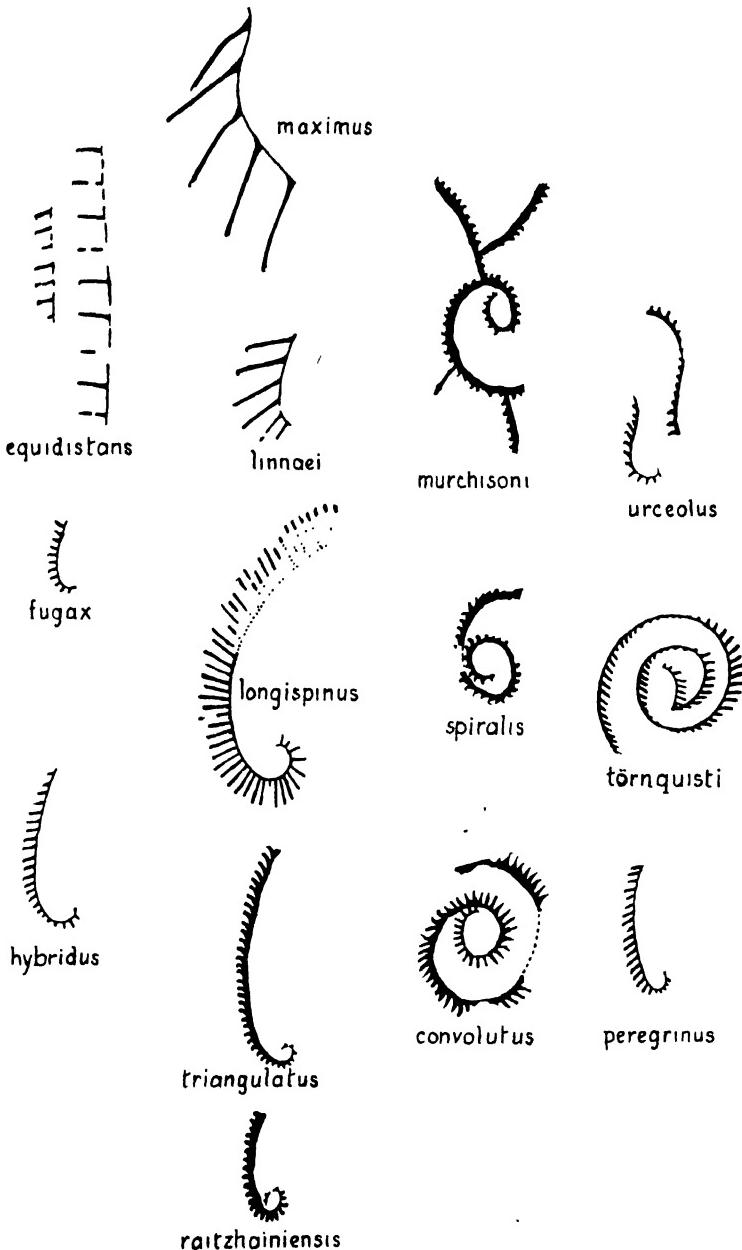
Fig. 4. Illustrations, natural size, of the members of the series indicated in Fig. 3. Figures of *R. peregrinus*, *M. törnquisti* and *M. urceolus* after Eisel (1912); the remaining figures from Elles and Wood (1902–18, plates).

isolation in the apertural region, the distal rim of the aperture being raised above the general level of the stipe, but this slight apertural isolation occurs in *Dimorphograptus decussatus* and is thus handed on from a pre-monograptid ancestor[1].

In the commonest forms of *Monograptus triangulatus* some 16 thecae are of the *Rastrites* type and even in the more distal part of the rhabdosome they are more drawn out and isolate than in *M. raitzhainiensis*. In *Rastrites longispinus* (Perner) the thecae are completely isolate and "linear," 3–5 mm. long, and occurring to the number of 7–8 in 10 mm. Each theca consists of a tube of approximately uniform width throughout its length, possessing a reflexed apertural termination. *R. linnaei* Barrande is a larger form, with 4–6 thecae in 10 mm., attaining in the mature portion of the rhabdosome a length of 8 mm.; their bases are triangular and the apertural ends are reflexed and slightly swollen. In the acme of this series, *R. maximus* Carruthers, the thecae may attain a length of 18 mm., with relatively small triangular bases and reflexed apertures, and are separated by interspaces of 10 mm. This species dies out leaving no descendants[2].

A second series, similar to this in many respects, is that leading to *Rastrites equidistans* Elles and Wood. At the base of this branch stands *R. hybridus* (Lapworth), which seems to be another of the descendants of *Monograptus triangulatus*, and, like *Rastrites longispinus* also, possesses numerous relatively short thecae, of uniform width and with a markedly reflexed aperture which here "constitutes a well-defined barb." *R. fugax* Barrande differs in having shorter and more widely spaced thecae, and in *R. equidistans* (which may not be a direct descendant of *R. fugax* though clearly related to this series) the thecae are separated by interspaces of 2 mm. and still measure only 2 mm. in length at the proximal end of the rhabdosome. Wider spacing of the thecae is not, in this series, accompanied by the extraordinary increase in thecal length which marks that of *R. maximus*.

There are numerous other species of *Rastrites*, many of which are probably members of some at present unrecognised lineages, while some may be offshoots from either of those described.

(2) *The* Cyrtograptus murchisoni *series* (Figs. 4 and 5).

A development of equal interest occurs in those series which, having proceeded some way along the isolate line, show a reversion to the original triangular type of theca, apparently by an almost exact reversal of the progressive evolution.

It seems to be agreed that *Monograptus convolutus* (Hisinger), of which fully the first twelve thecae are of *Rastrites* type and of which the thecae only begin to become

[1] According to Eisel, these species, both of *Rastrites* and "*Demirastrites*," are derived from *Rastrites spina* (Richter), itself a derivative of *Diversograptus attenuatus* (Manck); the ultimate origin of the series is not universally agreed upon and I have accepted the view current in Britain. It is not essential to the discussion, for the Continental view necessitates in an equal degree an ancestral form with triangular thecae. I take this opportunity of expressing my thanks to Dr Elfried Manck for the gift and loan of specimens of *R. spina* and to the authorities of the British Museum (Natural History) for the loan of other specimens of *Rastrites* and allied forms from Germany.

[2] It is possible that *Rastrites linnaei*, only doubtfully recognised in this country, is a parallel development to *R. maximus*, which is not recorded from Bohemia, and that the two forms are not terms of the same line of descent.
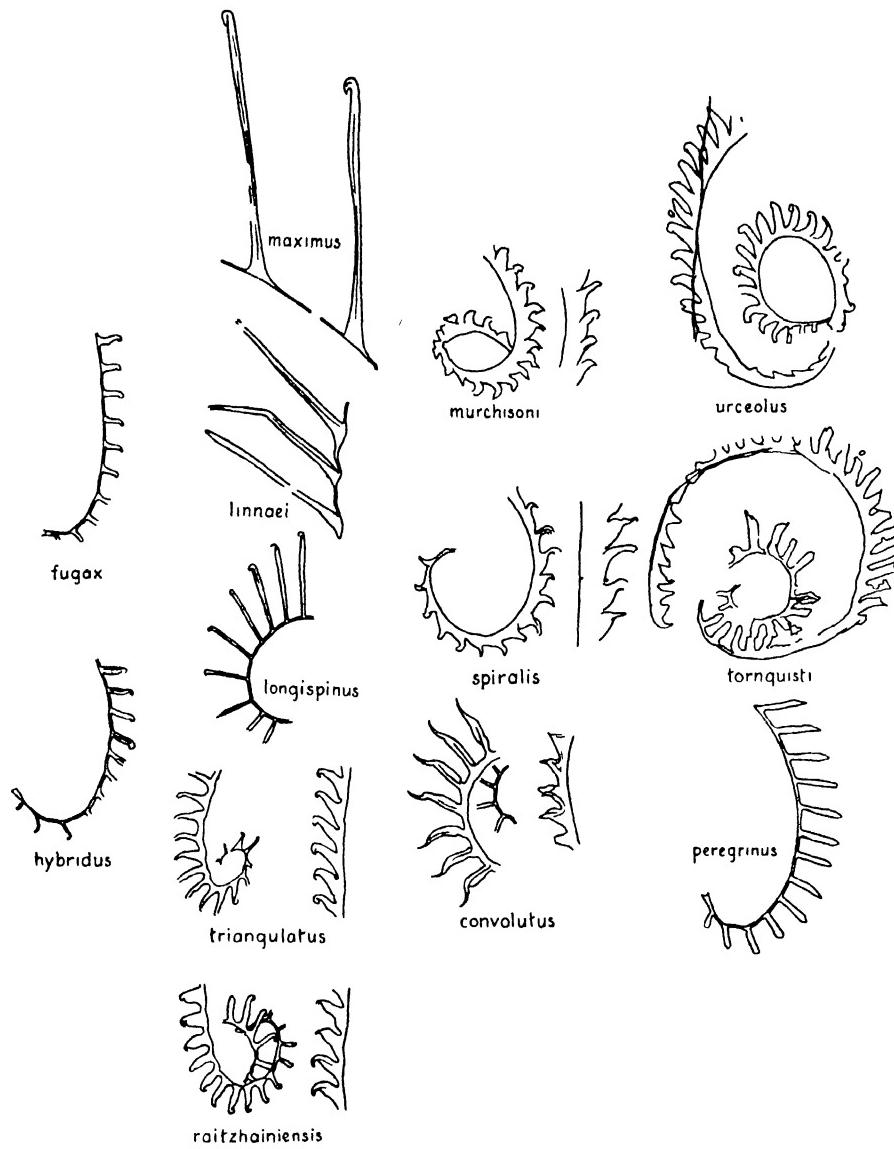
Fig. 5. Enlargements of portions of the rhabdosomes of species represented in Fig. 4, showing their thecal characters. All × 3 approx. *M. urceolus*, H 1521 (B.M.N.H.); *M. törnquisti*, H 1542 (B.M.N.H.); *R. peregrinus*, H 1366 (B.M.N.H.); the remaining figures from text-figures in Elles and Wood (1902–18).

really triangular after about twenty-four have been developed, is a descendant of *M. triangulatus* and is probably ancestral to *M. spiralis* (Geinitz) (Elles, 1922, p. 183; Eisel, 1912) where the thecae are, from the beginning of the rhabdosome, sub-triangular, isolate distally, and with a transversely elongated, reflexed, apertural termination. It is not unlikely that this species is ancestral to *Cyrtograptus murchisoni* Carruthers.

The other series, indicated by Eisel, would seem to have an even more marked reversal of evolution, since it is from a true *Rastrites* (*R. peregrinus* Barrande) that *Monograptus (Demirastrites) törnquisti* Eisel, and eventually *M. urceolus* Richter, are believed to be derived. The majority of the thecae in *M. törnquisti* are tubular, but are more closely set than in a true *Rastrites* and distally they are triangular; in *M. urceolus* some four or five thecae at the proximal end remain of *Rastrites* type.

Several other species, such as *Monograptus denticulatus* Törnquist, *M. decipiens* Törnquist, *M. pectinatus* Richter, and *M. nobilis* Törnquist, possess "biform" thecae and may be either in anagenetic or catagenetic stages.

IV. DISCUSSION OF THE EVOLUTION IN THE ISOLATE LINE.

(1) *Parallel evolution.*

What has been spoken of as the isolate or *Rastrites* line of evolution resolves itself into a number of different seriations, one group of which is progressive through-out, while the other shows regression before or just after the *Rastrites* characters are fully expressed. The accentuation of these *Rastrites* characters leads through *R. longispinus* to *R. maximus* and is paralleled by at least one other series (*R. hybridus—R. equidistans*) and to judge from the number of "unplaced" *Rastrites* species, future work will bring about the discovery of many more. Again, the reversal of evolution by which *Monograptus convolutus* and perhaps even *Cryptograptus murchisoni* is derived from a form like *M. triangulatus*, finds a parallel in the regressive *M. urceolus* series and possibly some of the other "biform" monograptids of this group (*Demirastrites*), among which the *Rastrites* type of theca seldom extends beyond the first five or six thecae of the rhabdosome.

It is not suggested that the series can be regarded as established lineages until more detailed field evidence is forthcoming, but that evolution along the lines indicated, both progressive and regressive, has occurred in numerous parallel lineages can scarcely be doubted, and such evolution may well be cited as an illus-tration of trends.

(2) *Widespread occurrence.*

Both the progressive and regressive series, but particularly the former, are represented in the Valentian (Lower Silurian) graptolite shales of widely separated areas, within which the evolution occurs practically simultaneously. Fig. 3 represents only the range of the species constituting the various series under consideration in Germany and the British Isles, but the majority of the species occur also in Scandi-navia (Sweden) and in Bohemia.

The palaeogeography of Valentian times is still in detail speculative, but it would appear that there existed a land promontory separating two large gulfs of the sea which lay over what is now Britain and Southern Scandinavia to the north, and Germany and Bohemia to the south (Jones, 1925, p. 134). Presumably the planktonic or pseudoplanktonic graptolites drifted into these gulfs from an open sea to the west, where they may have been undergoing their main development, but it is probable (*vide inf.*) that a certain amount of variation took place independently in the two embayments.

(3) *Local differences.*

If the species series from any two of these areas be compared in detail, it will be found that the time range of individual species may not be exactly the same and that the various members of a series are not identical in the two areas, but possess a local aspect.

The lack of perfect correspondence in the time ranges is illustrated in the case of the German and British series in Fig. 3. There is difficulty in making certain and exact correlation of the two series of zones on which the time ranges are measured, and future collecting will doubtless slightly modify the accepted zonal range, but the lack of agreement, if actual, may be taken to indicate that various evolutionary
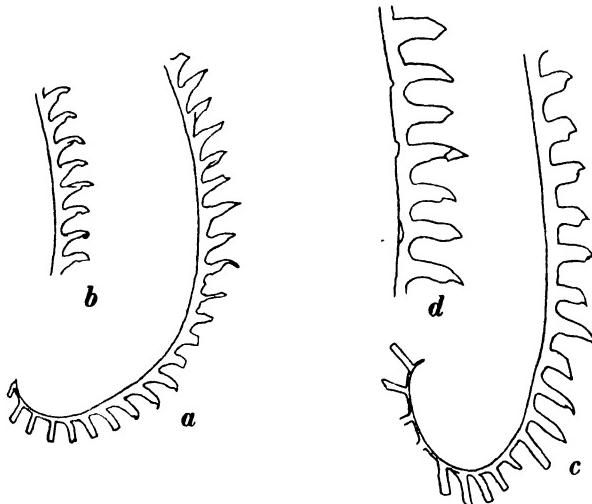
Fig. 6. *Monograptus triangulatus* (Harkness), × 3 approx. *a*, proximal end of rhabdosome, showing change in character of the thecae from tubular and isolate to triangular. Specimen 921, Sedgwick Museum. *b*, distal portion of rhabdosome, to illustrate the form of the triangular thecae. Specimen 902, Sedgwick Museum. *c*, proximal end of rhabdosome. Specimen H 1382, B.M.N.H. *d*, Distal portion of rhabdosome with triangular thecae, flattened by pressure. Specimen H 1353, B.M.N.H. *a*, *b*, British examples; *c*, *d*, German examples. Note the larger size and more widely spaced thecae of the German forms.

stages were reached at slightly different times in the different areas and persisted longer in some areas than in others.

Local differences in the form of the species claim a little more attention.

Absolute proof of these differences, and a true measure of their extent, could only be obtained by a comparison of material preserved in full relief (*e.g.* in limestones) from all the areas concerned, and such material is, of course, not available; but while some part of the observed differences is probably due to local differences in preservation, there is reason to suppose that underlying this there is an original difference in form which preservation has merely accentuated.

The British *Monograptus triangulatus*, for example, is a form of variable length, usually 3–4 cm., slightly curved, and with a maximum breadth of 2 mm.; the thecae number 8–10 in 10 mm. They are triangular in shape, in contact with each other but not overlapping, and the aperture is reflexed. In Germany, the species is usually considerably more than 3 cm. in length (one specimen recorded attaining a length of 9·5 cm.), slightly curved, and with a maximum breadth of 2–3 mm.; the thecae number 6–8 in 10 mm. The material is usually completely flattened and often somewhat distorted, but the characters of the thecae, as far as they can be made out, agree fully with those of the British form. The German examples are therefore larger and more robust; but whereas the robust British variety, *M. triangulatus* var. *major* has more closely set thecae than the normal form, those of the German *M. triangulatus* are even more widely spaced (Fig. 6).

Again, in the British *M. urceolus*, the stipe forms a loosely coiled helical spiral widening rapidly to a maximum breadth of 2 mm., and along which the thecae are set to the number of 11–12 in 10 mm. The German form of *M. urceolus* is a loose, often distorted spiral, stouter and more robust, and with the thecae numbering only 8–10 in 10 mm.

Among the various species of *Rastrites*, the differences may conveniently be tabulated:

	British			German		
	Thecal length	Number in 10 mm.	Inter-spaces	Thecal length	Number in 10 mm.	Inter-spaces
R. linnaei	8 (max.)	—	2–5	2–12	—	1·5–5
R. maximus	18 (max.)	—	10 (max.)	18 (max.)	—	3–5
R. hybridus	2	8–10	—	2	6–8	—
R. fugax	1	—	2	1	—	1–1 5
R. peregrinus	1–2·5	8–10	1	3–4	8–10	1

In Sweden and Bohemia, detailed measurements of the species again show slight differences.

This local aspect is probably to be accounted for by local conditions which might affect even the surface-living graptolites and suggests that modifications may have occurred in the isolated embayments, so that each area is to some extent a separate unit. It is not improbable that such local variations are of common occurrence among the graptolites and are responsible for much of the confusion which exists over specific identifications in different countries.

In this connection, reference may perhaps be made to an occurrence of similar local variations in the contemporaneous shallow-water fauna, as exemplified by

some brachiopods of the genus *Sowerbyella* (Jones, 1928). These differences can be
seen when we compare the species present on the northern and southern shores of
the trough or geosyncline extending across Great Britain towards Scandinavia. At
certain horizons, *undulate* forms occur in which the whole shell exhibits a more or
less regular radial folding, and each undulate form is more nearly related to some
normal form than to other undulate forms which occur at higher or lower horizons
(Jones, 1928). The normal forms are practically the same on the two sides of the
geosyncline (although slight differences may be seen even here), but the undulate
forms, while occurring at the same horizons, are of distinctly local type. The series
may be represented as follows:

North of geosyncline		South of geosyncline
Undulate forms	Normal forms	Undulate forms
	millinensis	
	parabola →	*compressa*
	↑	
	superstes →	*plicata*
	↑	
	duplicata	
duvali	↑	*undulata*
	mullochensis	
tricostata	↑	*tricostata*
	precursor	
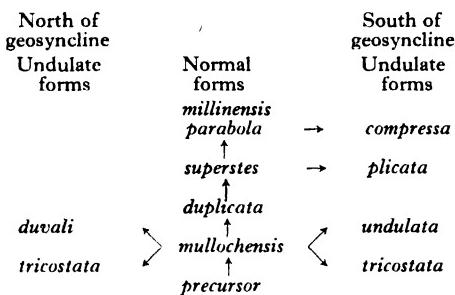
Although the species *duvali* is included in the synonymy with *undulata*, it is said
to be "not identical," and the Welsh or southern *tricostata* is distinctly smaller than
that of Girvan on the north.

(4) *Simultaneous progressive and regressive developments.*

That progressive and regressive series of development in the isolate line were
occurring together makes it difficult to see in this evolution any adaptive significance
or indeed any pronounced connection with the environment; for the external
causes, whatever they might have been, that would lead to the extreme isolation of
Rastrites maximus can scarcely be held responsible for the simultaneous return to
triangular thecae in other series such as those of *Monograptus spiralis* and *M. con-
volutus*, assuming, as I think we must at present, that these forms all lived side by
side.

(5) *Simultaneous operation of other trends.*

Not only are there several progressive and regressive branches of the isolate line,
but there occurred at the same time the beginnings of other lines of development in
other monograptid stocks, namely, the lobate line (reaching its acme in *Monograptus
lobiferus*, almost contemporaneously with that of the isolate line, in Zone 20) and
the hooked line (reaching its acme in *M. priodon* at the top of the Valentian and
passing through a steady retrogressive phase during the succeeding Wenlock period). •
And again there is the series, ending with *M. ultimus*, wherein the simple straight
thecal type, as far as we know, persists unchanged until the final extinction of the
graptolites.

V. PERIODICITY IN GRAPTOLITE EVOLUTION.

If the number of species existing at any given time be accepted as a general indication of the vitality of the race, it is possible to form some idea of the variation or constancy of this throughout the race history by plotting the number of species on a zonal scale.

When this is done for the graptolites, the resulting curve is not simple or uniform, but exhibits several clearly marked maxima indicating periods of great abundance of
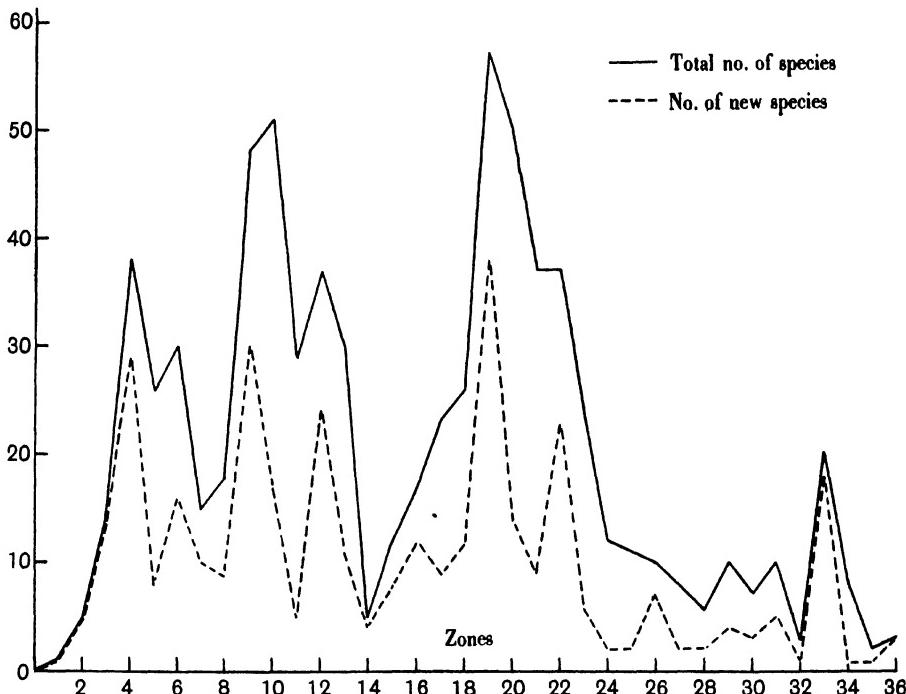

Fig. 7. Zonal distribution of graptolite species throughout Ordovician and Silurian times in the British Isles. The total number of species is indicated by the continuous line, the number of new species by the broken line. The numbers for the species in each zone have been obtained from Elles and Wood (1902–18, Table I). Zone 4, *Didymograptus extensus*; zone 9, *Nemagraptus gracilis*; zone 12, *Dicranograptus clingani*.

species, separated by periods in which there is relative scarcity. Such periods of abundance and scarcity might be the result simply of local conditions of deposition, corresponding to periods favourable and unfavourable to the life and preservation of a graptolite fauna in a limited area, but this view can scarcely be maintained when closely comparable curves are shown by the graptolite faunas of other areas. Curves for the Ordovician graptolite species of Sweden and North America, with maxima and minima at corresponding periods in the race history, are shown in Fig. 8 for comparison with the curve drawn for Great Britain (Fig. 7). The general occurrence

of these maxima suggests that they correspond to definite periods of evolutionary activity. The first maximum corresponds approximately to the *Dichograptus* fauna, the second to the *Leptograptus* and *Diplograptus* faunas, and the third to the
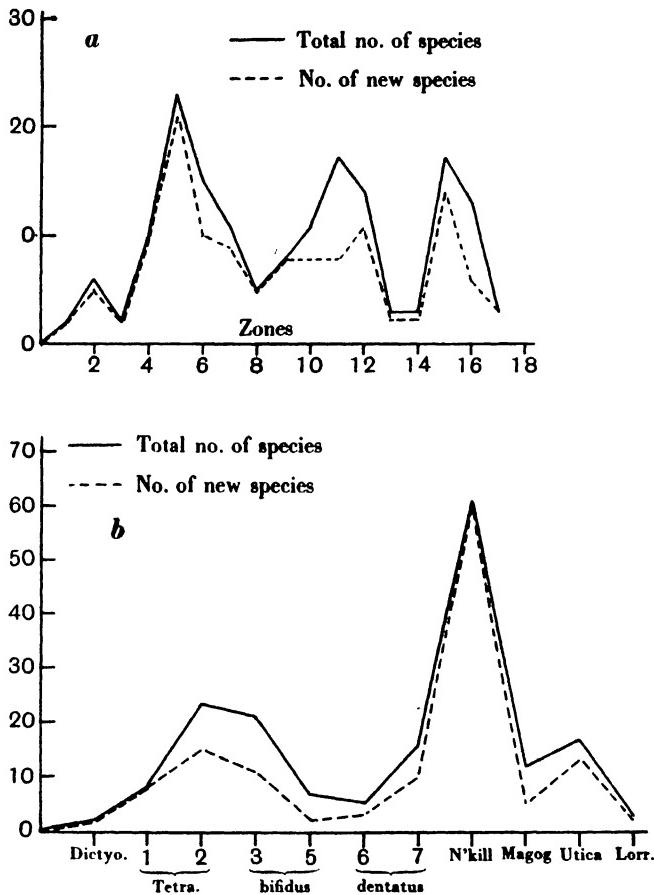

Fig. 8. Zonal distribution of graptolite species in the Ordovician of Sweden and eastern North America. *a. Sweden.* Based on data in Troedsson (1923). Zone 5, *Didymograptus balticus*; zone 12, *Nemagraptus gracilis*; zone 15, *Dicranograptus clingani*. *b. North America.* Based on data in Ruedemann, 1904. Zone 2 corresponds approximately to the *Didymograptus extensus* zone of Britain, the Normanskill Shale includes the *Nemagraptus gracilis* zone, and the Utica Shale includes the zone of *Dicranograptus nicholsoni* (slightly below the *Dicranograptus clingani* zone of Europe).

Monograptus fauna of Elles (1922). In other words, these definite periods of abundance of species are not only widespread (probably universal) but they coincide with the periods at which certain structural characters were generally attained by the group as a whole.

The first maximum, corresponding to the *Dichograptus* fauna, shows two peaks, of which the second (later) one is probably connected with the retarded development of the pendent series of didymograptids.

The second maximum has again two peaks, the first corresponding to the *Leptograptus* fauna (zones 8–10) and the second to the *Diplograptus* fauna (zones 11–15). These do not coincide with any pronounced change in the generic composition of the fauna and indeed Dr Elles, while commenting (1922, p. 194) upon the important numerical abundance of individuals of diplograptids in the second fauna, has already remarked that there is no new element introduced in this *Diplograptus* fauna. There is a double maximum in the distribution curves for most of the genera, but whilst the curves for *Diplograptus* (*s.l.*) and *Climacograptus* are rather irregular, the leptograptids show it very distinctly (Fig. 9). The first maximum corresponds to the more elaborate cell phase of *Leptograptus* and *Nemagraptus*, the second to the renewed activity which appears to have followed the return to the simpler thecal
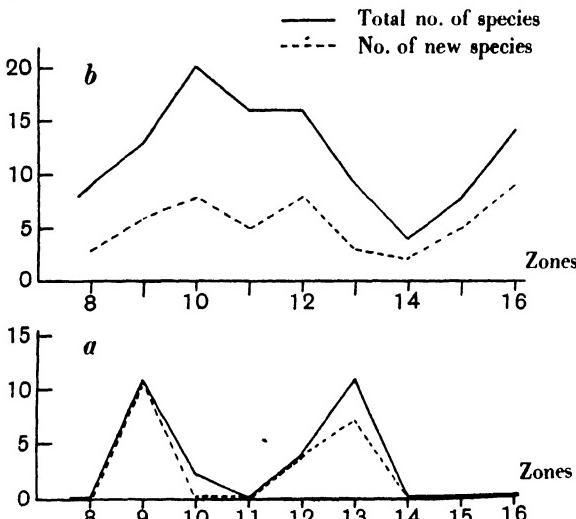

Fig. 9. Zonal distribution of species of leptograptids (*a*) and diplograptids (*b*) in the Ordovician of Great Britain (zones 8–15), showing the pronounced double curve for the leptograptids.

type in *Pleurograptus* and *Amphigraptus*. Similar results are obtainable from the data of the Swedish graptolites (as given, for example, by Troedsson, 1923), but the number of species is fewer and the curves are thus less reliable.

The third maximum (Fig. 7) corresponds to the earlier portion of the *Monograptus* fauna, a period during which most of the thecal elaboration was initiated, and again there is a period of rejuvenation and renewed activity in the Lower Ludlow (zones 32–35) following the return to simpler thecae. It has not been possible to obtain the necessary data to construct the corresponding curves for the Silurian graptolites of other areas.

There is no evidence that general conditions over a wide area varied sufficiently to account for these periods of activity. Either we must conclude that there is a connection between the vitality of the race and the elaboration periods (as suggested by Spencer for the Asteroids) suggesting some internal factor in evolutionary

activity, or that certain grades were what might be termed "successful structural grades" at which numerous species were rapidly evolved as they were successively reached by the group as a whole. The latter interpretation does not readily account for the falling off in numbers of species, which invariably follows rapidly after their "bursts" of development, or "expression points," nor for what have been called the rejuvenation modes of the leptograptids and monograptids.

VI. RELATION OF TRENDS TO ENVIRONMENT.

The work of Rowe, Woods (1912), Spencer, and Lang (1917, 1921, 1923 *a*) on the Chalk micrasters, *Inocerami*, Asteroidea and Polyzoa respectively, has made us familiar with the occurrence of evolution under almost constant environmental conditions. Although the Chalk was deposited under conditions which were peculiar and are perhaps not fully understood, yet, as Woods (1897) has pointed out, "in its original soft state, the chalk ooze must have been, so far as animal life was concerned, uniform in character throughout the Chalk period" and the physical conditions for this precipitation must have remained practically constant. Various bands of "rock"; the Top Rock, Chalk Rock, and others lower in the succession—which carry a fauna containing abundant gasteropods, ammonoids and lamellibranchs, are believed to mark periods of shallowing, but these shallowings were of relatively short duration and on the return to deeper conditions the normal Chalk fauna becomes again dominant. The "zoological break" of Rowe, which separates the high-zonal from the low-zonal series of micrasters occurs about one-third the way up in the *Micraster cor-anguinum* Zone (well above the Top Rock horizon) and one of the two important developments in the Asteroidea also occurs at about the same level, similarly unrelated to any recognisable lithological change; while the "faunal breaks" in the polyzoan assemblages are slightly higher stratigraphically than those of the Asteroidea (Lang, 1917, p. 224). In all these series, there is nothing in the evolutionary history corresponding to the few and slight lithological changes known to occur.

In the *Sowerbyella* series referred to above, it seems possible that comparable evolution took place under somewhat different environmental conditions on the two sides of the geosyncline (p. 325). It is not so much that the lithology of the sediments is unlike (in both cases the rocks are for the most part shallow-water mudstones and sandstones) but that the associated fauna on the northern and southern shores of the trough have scarcely a species in common, and in consequence the biological environment must have been entirely different. The *Sowerbyella* species, like their associates, are shallow-water types which probably could not have migrated directly across the deeper central portion of the trough, and must therefore either have migrated round the shores, or have been introduced from some outlying area in which their main development was taking place, or have evolved almost independently on the two sides. One would expect that a periodic immigration, or a migration around the coastal areas, would have been accompanied by the introduction of many more species common to both sides of the geosyncline than is the case; but

whatever the explanation, it remains of importance to note that the trends of evolution on the two shores are the same and that only local variations are exhibited.

In the monograptid evolution described in the preceding pages, it is probable that the local aspect which characterises the different series in the various areas where they occur may have been due to the direct influence of local conditions, accentuated by inbreeding (assuming sexual generations to have existed). But these differences are superimposed on main lines or trends of thecal elaboration in which it is difficult to recognise at present either adaptation or chance selection. There would appear to be analogous instances of such local influence among the Asteroidea and the Polyzoa (Lang, 1917, pp. 205, 218), where again the principal lines of evolution are so clearly distinct from local variations.

It would seem therefore that we may recognise as distinct in all these series the comparatively small changes due to the direct influence of environmental factors, such, probably, as temperature, salinity, food-supply, etc., and the slower and more profound changes by which the race achieves, sometimes a more perfect adaptation to its mode of life (judging by its success) and sometimes such disharmony as may lead to its extinction.

VII. POSSIBLE EXPLANATIONS OF TRENDS.

Probably the term Programme-Evolution has been used to cover a number of slightly different types of evolution, having in common the fact that they are directional and occur in parallel lineages, for which no one explanation will suffice. Before passing to the possible origin of the process in the graptolites, it may be permissible therefore to consider briefly the views that have been expressed with regard to trends in other groups of animals.

A point usually stressed, nor unduly so, is that in programme-evolution various characters in independent lineages are frequently carried to such an exaggerated state of development as apparently to be harmful to the organism and to lead to the extinction of the forms concerned (*e.g.* lobation in the monograptids). Hence, for the greater number of cases, natural selection has been deemed an inadequate explanation, although attempts have been made to reconcile this theory to the facts. Thus Dendy (1912) considered that natural selection, by selecting individuals in which some structure (*e.g.* horns) was most developed, might, *ipso facto*, minimise the secretion of the hormones which inhibited the development of the structures in question, for in the selected individuals these hormones would be least active. By this means the lineage would become progressively deficient in the inhibiting secretion, and would finally be exterminated by the uncontrolled growth of the very structures which at first possessed selective value[1].

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[1] It has recently been suggested by J. S. Huxley (*Problems of Relative Growth*, London, 1932) that such orthogenetic series as those of the titanotheres are a natural consequence of increase in general bodily size, and that assuming that "there existed in the ancestor of the four lines of descent the hereditary basis of growth-mechanism for a frontal horn, and that increase in size up to a certain limit was advantageous for titanotheres in general, as would seem inherently probable, then the results follow without any need for invoking orthogenesis" (p. 218).

Duerden (1919) concluded from his study of the ostrich, which shows similar degenerate features over a wide area of varied climatic and geographical conditions, that "only something inherent within the organism itself and beyond all varying somatic responses, could meet demands so continuous and consistent," and that probably the changes are to be interpreted in terms of germinal senescence. The explanation which was arrived at by Fenton (1931) from a study of the Devonian brachiopod *Spirifer*, is analogous. On his view, a decrease in the metabolic rate accompanying phyletic senescence gives rise to morphological and physiological changes which are limited in type, which show distinct (though not necessarily direct) evolutionary trends, and which are cumulative. "This metabolic decrease itself pursues a trend which, though it may be delayed or diminished, apparently is not halted, so that its ultimate termination is senility and extinction."

In all these cases, the directive force of the trends is attributed to an essentially physiological basis. The possibility of direct environmental influence has to be rejected in the majority of cases, although there are a few in which it has been looked to as a possible cause of trends. Thus in an area of rapid sedimentation, a sedentary animal might by rapid growth raise itself above the accumulating sediment, and this has been suggested as the origin of the elongation (caninoid trend) and decreased septal construction (amplexoid trend) in rugose corals; of the elongation of the attached valve of the Rudistes; and perhaps too of the grypheate trend of *Ostrea*. But in general, such direct influences are seen to be capable only of producing geographical races, and, among the graptolites at least, there is no evidence that these races ever developed into distinct species.

Linkage, again, affords a possible explanation of variation in such characters as shell ornament, which seem neither harmful nor beneficial to the organism, but it usually involves a hypothetical connection with selective characters whose existence is entirely assumed, and it is not applicable to many series.

Finally, where structure is undergoing change, habit must in general be changing too, and if such changes occur while the environmental conditions are constant and uniform, it is possible that change in habit was the *primum mobile*.

Regarding the main trends of the graptolites, it has been suggested that in two of them adaptation may have played some part, and they have generally been assigned an adaptive origin. These two are the stipe-reduction and the scandent direction of growth trends. But when we consider all the facts at present known, it is by no means certain that this explanation is sufficient. That external factors (*e.g.* balance) controlled the manner of stipe-reduction, so as to produce at each stable stage a symmetrical rhabdosome is extremely probable; but that the reduction itself was a selective modification ensuring an adequate food supply to the remaining "zooids" is more doubtful. Firstly, it affords no explanation of the progressive delay in the production of "double buds" (*i.e.* of dichotomy) which apparently occurs through-out the diplograptids as a continuation of the stipe-reduction trend, and secondly, the question of the very marked diminution in number of the species after the os-tensibly successful two-stiped stage was reached, remains untouched. Moreover, the second period of abundance of the leptograptids corresponds with the reversion

to the many branched rhabdosomes of such genera as *Amphigraptus* and *Pleurograptus*. The same objection applies in respect of the diminished evolutionary activity at the end of the diplograptid fauna; not only is it difficult to see any selective value in the early stages of the scandent direction of growth trend (*i.e.* in a reclined as opposed to a horizontal rhabdosome, or in a *Dicranograptus* with a very short biserial portion, as compared with a *Dicellograptus*), but the presumably successful scandent diplograptids diminish conspicuously in numbers considerably earlier than the first monograptid appears to offer any competition.

In such of the thecal elaboration trends as have been considered here, it has been suggested that they are not due to any direct environmental influence (which is, however, seen in local geographical variations in each series); and against the view that they are adaptive is the fact that at least three types are initiated simultaneously, all under apparently the same conditions of environment, and that regular regressive series also occur. Either they are not adaptive, or we must assume that the trends represent forms which did in fact live under slightly different conditions of environment (*e.g.* suspended in some way at different depths in the water and perhaps possessing some specialised feeding mechanism) for which view there is certainly no evidence. Again, when two of these trends have become extinct and the third has reverted to the condition of simple thecae, so that all the monograptids are once more simple-celled forms, there is, in Great Britain at least, another period of activity in which numbers of new species appear, just before the final extinction of the group.

While rejecting such provisional interpretations as have already been advanced, it seems at present impossible to offer any alternative explanation of the origin of these trends. In general, certain characters have, of course, a very limited range of variation. Particularly is this true of dimension ratios and the "trends" based on these. On such lines, it might perhaps be predicted that among the monograptids, the simple-celled, sigmoid, climacograptoid, isolate, hooked, and lobate series will all in time be found to occur as separate trends. The organism has certain inherent possibilities and certain inherent limitations.

Yet if we regard the phenomenon of programme-evolution as an expression of the inherent limitations of a race in respect of possible variation, no explanation is offered of the real cause of its directional character. This may have a different significance in different instances, for sometimes it seems to be adaptive and sometimes to have an interpretation in terms of racial senescence, though this may be only one aspect of a deeper cause. As concerns the graptolites, I offer the view that there is very little evidence that any of the trends originated in response to external conditions; they seem, on the contrary, far more readily referable to some internal factor. In favouring some physiological explanation, however, it seems scarcely feasible to look to any form of degeneration as the mainspring of the whole evolutionary development of a group, and there is, moreover, evidence suggesting several periods of progression and regression, of virility and degeneracy, both in the group as a whole and in the more detailed histories of separate lineages.

VIII. SUMMARY.

1. As an illustration of the occurrence of programme-evolution among the graptolites, some of the series included within the "isolate" line of thecal elaboration in the monograptids have been selected.

2. These are represented in the Lower Silurian (Valentian) rocks of Sweden, Britain, Germany and Bohemia. When the species constituting these series in any one area are compared with those of another, as is attempted here for the British and German forms, they are found often to exhibit slight differences in form (although occupying the same relative positions in precisely comparable evolutionary series), and possibly also in time-range. It is suggested that this local aspect is probably to be attributed largely to direct environmental influence.

3. Against the view that the thecal elaboration is adaptive, it is pointed out that several distinct progressive and regressive lines of development, not only in the isolate line, but also in the hooked and lobate lines, occur simultaneously (while simple unmodified thecal types also persist), and all these are represented by species which, as far as can be ascertained at present, lived under the same environmental conditions.

4. There is evidence of certain definite periods of evolutionary activity (represented by a marked abundance of species and particularly of new species), again suggesting the operation of what may be referred to as some "internal" factor in the evolution of the race. None of the explanations which has been advanced in the interpretation of similar series in other groups of animals seems applicable here, but no alternative is offered.

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THE *IN VITRO* CULTIVATION OF FILTERABLE VIRUSES

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CONTENTS.

I. INTRODUCTION.

MANY attempts have been made to cultivate under artificial conditions the viruses affecting plants and animals. There is, as yet, no record of undoubtedly successful *in vitro* cultivation of the plant viruses. The literature, however, abounds with records claiming success with those affecting animals. Early work reveals much confusing evidence in favour of certain frankly bacterial agents as the cause of certain virus diseases. None of these have withstood the criticism which modern methods of virus research and increased knowledge of the nature and properties of the filterable viruses have brought to bear on them. The ability of a virus to propagate itself indefinitely under conditions of artificial culture is of the greatest importance for a decision as to the nature of viruses. Two views regarding the nature of filterable viruses are commonly held. A virus may be a non-living active substance, possibly toxin or enzyme in nature, capable when brought into contact with susceptible cells of so affecting their metabolism that more substance of the same kind is produced. The second, and more widely-held, theory postulates a living particulate nature similar to that of the micro-organisms. It is a basic fact that viruses multiply illimitably in the tissues of affected hosts. The simplest explanation of this is to assume them to be living organisms, for on this view the fact of multiplication is explained naturally. The proof of multiplication is an integral part of such a theory and as such is demanded as perhaps the most important link in the chain of evidence that has gradually grown in favour of the living character of the viruses.

II. THE NATURE OF FILTERABLE VIRUSES.

If the living organismal nature of filterable viruses be not accepted there is no clearly formulated alternative explanation. Sanfelice in 1914 suggested that a virus is a non-living poison elaborated by affected cells capable of attacking new cells which in turn produced more poison. Thus the undoubted multiplication of viruses is considered to be a function of the living cell, and in this way the difficulties presented by multiplication are dealt with. It is admittedly difficult to cultivate the filterable viruses in the absence of living susceptible cells; and it is this difficulty more than any other that has perpetuated the non-living concept of viruses.

If viruses are living objects they must be particulate. Barnard and Elford (1932) have studied by ultra-violet light photography the elementary bodies found in infectious ectromelia and those described in vaccinia by Paschen in 1906. Improvements in technical methods of virus research have shown that the elementary bodies in vaccinia readily demonstrated by appropriate staining methods are invariably found in enormous numbers not only in the characteristic skin exanthem, but also in those gross organ lesions which may follow the inoculation of highly potent virus in experimental animals.

Bechhold and Schlesinger (1931), in an attempt to estimate the size of the infective virus particle in vaccinia by physical calculations based on high speed centrifugalisation experiments with virus suspensions, decided that the diameter of the particle was $0.21-0.23\mu$. Many recorded measurements of the stained elementary bodies are in close agreement with this figure, viz. $0.2-0.25\mu$. Filtration through specially prepared collodion membranes of measured pore diameter has shown that the size of virus particles may be estimated within close limits and individual viruses separated from a mixture by selective filtration. Elford and Andrewes (1932) have estimated that the size of the virus particle in a testicular strain of vaccinia is $0.125-0.175\mu$. Barnard and Elford (1932) found the particle size of infective ectromelia to be $0.1-0.15\mu$. The virus of foot-and-mouth disease was shown by Galloway and Elford (1931) to have a diameter of $8-12\mu\mu$.

Ledingham (1931, 1932) has shown that pure suspensions of elementary bodies in vaccinia and fowl-pox can be obtained in formalised saline, and that these are specifically agglutinated by the serum of vaccinated animals. The agglutination can readily be followed in hanging-drop preparations, and the stained clumps are found to consist entirely of elementary bodies. An analogy of the elementary bodies with true bacteria is provided by the fact that after a single dose of virus agglutinins appear in the serum, rise to a maximum and slowly fall in precisely the same manner as do agglutinins in response to any bacterial antigen.

Nauck and Paschen (1932) have recently shown that in cultures of vaccinia virus grown *in vitro*, using testicle tissue, the increase in numbers of elementary bodies is coincidental with a rise in titre of those cultures as shown by animal experiment. The presence of numerous elementary bodies in stained preparations of vaccinia cultures grown by Eagles and McClean (1931) in a cell-free medium is additional evidence of the nature of these bodies.

Eagles and Ledingham (1932), using high-speed centrifugalisation, were able to deplete Berkefeld " V " filtrates of vaccinia virus almost entirely of their virus content and to recover the great bulk of the virus in deposits consisting mainly of elementary bodies. These deposits retained their potency after repeated washings in saline, while the saline menstruum in which the bodies were suspended was entirely free of virus activity.

Amies (1932) has obtained elementary bodies of variola in pure suspension and has shown that these are agglutinated by the serum of patients convalescent from either the mild or severe form of smallpox. The reaction is specific, as appears from the fact that vaccinia elementary bodies are not agglutinated even in the highest concentrations of such sera.

Elementary bodies have been studied by staining and other methods in molluscum contagiosum, ectromelia, fowl-pox and psittacosis, and a great deal of evidence of an experimental nature has been produced that they represent the actual infective agents in these diseases.

The exact nature of cell inclusions in filterable viruses is not clear. There is a growing opinion, however, that they are not products of cell activity resulting from stimulation by the virus agent but are collections of elementary bodies within the cell. Woodruff and Goodpasture (1930) have shown that the specific cellular inclusion, the Bollinger body, of fowl-pox is largely composed of uniform granules which correspond in their morphology, numbers and staining reaction with Borrel bodies demonstrated in smear preparations. These inclusions were found after isolating and repeated washing to produce typical infections on inoculation. The specific inclusions in molluscum contagiosum have been found by Goodpasture and Woodruff (1931) to correspond in size, numbers and staining reactions with the Borrel body of fowl-pox though they were unable to prove their infectiveness owing to technical difficulties. Bedson and Bland (1932), in a study of the morphology of psittacosis virus, described a developmental cycle. It appeared that the elementary body represented the final stage. The elementary bodies after having gained access to a cell developed amoeboid forms which, coalescing, formed a plaque or plasmodium. This in turn divided into a number of portions of equal size in which the virus is revealed as a spherical body packed with oval segments. These divide and subdivide giving rise to the elementary body.

III. THE CULTIVATION OF VACCINIA VIRUS.

It is not surprising that successful *in vitro* cultivation was first realised with vaccinia virus, for strains may be obtained with ease which are free from bacterial contamination. Small amounts of virus may be detected with practical certainty by inoculation of common laboratory animals, and research along lines other than cultivation has been persistent over a long period. The ability of vaccinia virus to reproduce itself in culture may now be considered as established in view of the ample confirmation it has received. Numerous early attempts to cultivate the virus without tissue culture were made, but these remain unconfirmed. In 1913 Stein-

hardt, Israeli and Lambert, and in 1914, Steinhardt and Lambert, with a sterile dialysate of glycerinated calf-lymph as seed reported success using rabbit and guinea-pig cornea in tissue culture and carried the virus through the third generation in subculture. They believed living tissue to be essential to virus growth. Parker (1923-4) and Parker and Nye (1925) propagated the virus through a number of generations and obtained an increase of 51,000 times. Small pieces of vaccine-infected testicle were implanted in rabbit plasma where active proliferation of the cells occurred regularly. Carrel and Rivers (1927) used minced 7-10 day chick embryos in Tyrode's solution and hen's plasma. A definite increase in virus content was obtained. Haagen (1928) employed rabbit testicle and spleen incorporated on cover slips in rabbit plasma. Thirty-seven subcultures were made and a thousand-fold increase obtained. Eagles and McClean (1929) repeated the experiments of Carrel and Rivers, using both a pure dermal and a neuro-testicular strain of virus. Multiplication of virus was definite but irregular, in that only occasional flasks in an experiment showed increase although conditions were apparently similar in all. There was evidence that embryo brain tissue was more suitable than whole embryo in that greater multiplication resulted from its use. They pointed out that active cell proliferation is not essential to virus growth nor is it an indication that increase in virus will occur.

A definite advance in simplifying the culture of vaccinia virus, and of viruses generally, was realised when Maitland and Maitland (1928) and Maitland and Laing (1930) obtained excellent growth of virus using fresh minced adult kidney either from hen or rabbit in a fluid menstruum of Tyrode's solution and fresh serum. It was at first assumed that the virus had grown in non-living tissue, but Rivers, Haagen and Muckenfuss (1929a) pointed out that the kidney cells remained alive for 5-6 days in this medium. Eagles and McClean (1930), studying the comparative value of Carrel and Rivers' technique and the Maitland kidney medium, concluded that while excellent virus growth could be obtained with either method, irregularity occurred equally in both. The undoubted advantage of the Maitland medium lay in its simplicity.

It was natural that the practical aspect of "culture" virus of vaccinia should be investigated for several reasons. "Culture" virus is desirable because of its bac-teriological sterility, the simplicity and inexpensiveness of its production and the fact that animals are required only for the final potency tests. Three main difficulties are apt to be encountered: the irregularity in increase, the failure to produce large quantities of potent virus at will, and the tendency of the virus to deteriorate rapidly under ordinary conditions. Li and Rivers (1930), using a neuro-vaccine as seed with chick embryo tissue in Tyrode's solution, obtained regular multiplication. They realised, however, that neuro-vaccine is undesirable in human vaccination. Rivers (1931), therefore, used a pure dermal strain, and with the same methods of culture obtained a culture virus which produced in monkeys, rabbits and man typical vaccinial reactions, rendering them refractory to infection with ordinary vaccine virus. The virus in 50 per cent. glycerine at $+ 2 \cdot 5°$ C. remained potent for at least a month.

IV. OTHER VIRUSES WHICH HAVE BEEN CULTIVATED
BY *IN VITRO* METHODS.

The position of virus cultivation at the present time has been reviewed at length by Eagles (1932). From this emerges clearly the fact that although a relatively large number of viruses have been cultured on artificial media the conditions necessary to success are not well understood. It may be stated broadly that although much research has been carried out success has tended rather more toward increasing the number of cultivable viruses than to an intensive study of the processes involved. Moreover, it is noteworthy that the methods successfully employed in vaccinia have been, with minor alterations in individual cases, successful in other viruses. Most investigators claim that living cells are an essential part of any medium employed. They need not be in a state of active proliferation. A brief survey of the methods found satisfactory in viruses other than vaccinia will serve to indicate the general lines along which virus cultivation has proceeded.

Findlay (1928) found that tissue-culture methods used by Carrel and Rivers in vaccinia enabled him to obtain survival and multiplication of the virus of fowl-pox. He succeeded in carrying the virus through four generations with a definite increase at each subculture. Glover (1929, 1930), working with a pigeon strain of fowl-pox, found also that active tissue proliferation was necessary to growth of virus in a series of five subcultures.

The virus of foot-and-mouth disease has attracted considerable attention. The earlier attempts to cultivate this virus were many and unsuccessful. It was not until 1931, when Maitland and Maitland employed tissue culture as a medium, that undoubted cultivation was realised. They found chick embryo in hen plasma unsuitable, apparently on account of a species peculiarity. Pads, lips, tongue and hairy skin of embryo guinea-pig in fresh guinea-pig serum gave excellent growth. The virus was carried through seventeen successive cultures, representing in the final subculture a dilution of 4.8×10^{39} times the original virus. Their findings were confirmed by Hecke (1930, 1931) in essential respects.

Nigg and Landsteiner (1932) successfully cultivated the rickettsia of typhus fever, using either the technique of Rivers, Haagen and Muckenfuss (1929a) in large tubes containing normal tunica from half-grown guinea-pigs soaked in rickettsia suspension and a small amount of heparinised guinea-pig plasma, coagulation being effected by Ringer's solution extracts of normal guinea-pig spleen, or the Maitland method for cultivating vaccinia. Serum was an essential part of the medium.

Haagen and Theiler (1932) found tissue culture suitable for propagating the virus of yellow fever. Chicken embryo was used extensively and gave better results than kidney and testes of guinea-pigs. The virus was carried through twenty subcultures, the final subculture representing a dilution of 5×10^{15} times the original virus material without loss in titre.

Virus III of rabbits has been cultivated by Andrewes (1929a, b), using either tissue culture in the form of rabbit testis in plasma or the surviving tissue method

of the Maitlands in vaccinia. Both were satisfactory. The intranuclear inclusions associated with infection of this virus were readily demonstrated in cultures by either method. Topaccio and Hyde (1932) repeated Andrewes' work in all its essentials.

Tissue-culture methods have been employed successfully with herpes virus by Rivers, Haagen and Muckenfuss (1929b), Gildemeister, Haagen and Scheele (1929), Andrewes (1930) and Saddington (1932). The technique used by these investigators did not vary in any essential manner.

The position with regard to poliomyelitis is not so satisfactory. A number of workers have reported successful cultivation of the virus in the form of the globoid bodies described by Flexner and Noguchi (1913), but confirmation and contradiction being almost equal, no definite conclusions can yet be formed.

A number of viruses, including rabies, mumps, measles and encephalitis lethargica, have been reported as cultivable. But the evidence relating to their cultivation is unsatisfactory, and, indeed, with the exception of rabies the nature of the causative agent is not established.

V. THE RATIONALE OF VIRUS CULTURE.

From the examples of viruses which have been cultivated under artificial conditions it is possible to discuss in a general way the conditions under which a virus may reproduce itself. It may safely be assumed that the filterable viruses may, under suitable conditions, be cultivated *in vitro*. The application of tissue culture and its modifications to the problem of the cultivation of vaccinia virus has stimulated the investigation by similar methods of other viruses with similar successful results.

We have, however, learned surprisingly little regarding the conditions necessary for virus culture. It is generally stated that viruses multiply only in the presence of living susceptible cells and that they gradually deteriorate, under cultural conditions, when these are no longer viable. This broad general statement is likely to be premature, for, although the cultivation of viruses has been studied by an increasing number of workers, very few have done so in the intensive fashion necessary to overcome inevitable initial difficulties. In the case of vaccinia, Maitland, Laing and Lyth (1932) suggest that success or failure in cultivation depends on a number of factors, the most significant of which are the respiratory activity of cells, which varies considerably in tissues from different organs, and the free access of oxygen to these tissues. They also point out that a proper ionic composition of the medium is essential, since only under such conditions do cells survive. This hypothesis assumes that living cells are essential to virus culture. It is difficult to reconcile their assumption with the experience of Dochez, Mills and Kneeland (1931) and others in the cultivation of the virus of the common cold. Here the virus appears to multiply under anaerobic conditions. If the evidence which has accumulated and which points inevitably to the organismal nature of certain viruses already discussed be accepted it is only reasonable to suppose that individual viruses may vary considerably in their cultural requirements as do the bacteria.

The cell requirements of viruses under culture conditions is still an open question. Eagles and McClean (1930) showed, in a short series of subcultures, that the supernatant fluid after centrifugalisation of fresh minced rabbit kidney and Tyrode's solution was capable of promoting the growth of vaccinia virus. This fluid contained only a trace of cell *débris* as shown by stained smears and hanging-drop preparations. In a later communication (1931) the possibility of cultivating vaccinia in a cell-free medium was more thoroughly investigated. The base of the cell-free medium was, as previously, the supernatant fluid from centrifugalised minced fresh kidney and Tyrode's solution. This so-called "kidney extract" was apparently free of cells. An increase of 10^{43} times the original virus inoculum was obtained in the course of thirteen subcultures. A short series of four subcultures indicated that even after passage through a Chamberland L_2 candle the extract favoured the growth of virus. In the filtered medium the presence of occasional cells or cell fragments, which might conceivably favour multiplication of the virus, was definitely excluded. Eagles and Kordi (1932) further investigated the possibilities of cell-free culture. In a successful series of subcultures great care was taken to obtain, by the use of hypertonic saline and prolonged grinding of the kidney, an extract rich in cell substances though free of actual cells. The author has recently extended this work and found that the extract while free of cells is apparently extremely rich in cell granules. It is important that the medium be freshly prepared for each culture. Great difficulty was experienced in establishing the virus in culture and considerable irregularity in growth of virus was experienced.

The occurrence of elementary bodies in culture of vaccinia has already been indicated. The importance of seeing growth in cell-free medium is apparent when one realises that these bodies must proliferate freely in the medium. The crucial nature of cultivation in cell-free medium lies in the consideration of the relation of elementary bodies to cell inclusions. It is at once apparent that if the inclusion bodies that are typical of certain virus diseases are an essential phase in virus multiplication it is impossible for viruses to grow in any medium devoid of cells. Reference has been made to the relation of the inclusion bodies in fowl-pox and vaccinia to the elementary bodies typical of these viruses. It is not definitely established, however, that the elementary bodies found free in stained smear preparations are derived solely from the mechanical breaking up of the inclusion bodies. The experiments of Bedson and Bland (1932) point to an intracellular existence during the growth cycle of psittacosis virus. In tissue cultures of mouse spleen a definite increase in virus was obtained and the entire developmental cycle observed. In tissue culture no virus was seen outside the cells; intracellular conditions were apparently essential to virus growth. There is strong evidence that the viruses of vaccinia, fowl-pox and molluscum contagiosum are cytotropic in the usual sense of having an especial affinity for cells. Goodpasture, Woodruff and Buddingh (1932) are of the opinion, based on infection experiments with the chorio-allantoic membrane of the developing embryo of the fowl, that they are also cytotrophic in the sense that they require under natural conditions an intracellular environment for their growth. It may, however, be possible that whole cells are not essential and

that the granular material from broken-up cells may supply conditions approximating intracellular conditions.

It seems probable that the actual nature of the multiplication of viruses will not be well understood until simpler methods of culture are employed. Viruses whose particles lie within the size capable of resolution under the microscope may be observed in unstained hanging-drop preparations with dark-ground illumination. But the nature of the multiplication of these particles is not known. If it were possible to cultivate the larger sized viruses on solid media a definite advance would be made and the growing opinion that they are really only extremely minute organisms, and possibly that by reason of size are more difficult to cultivate than the bacteria, would be more readily accepted. To realise fully the analogy with the bacteria, colony formation must be observed. Reports of cultivation of viruses on solid media are not wanting. The earlier literature on vaccinia reveals a number of instances claiming some bacterial agent as the causative organisms. Frosch and Dahmen (1924) described colonial growth of the virus of foot-and-mouth disease. But careful investigation by a number of later workers failed to substantiate their findings.

The case of the so-called "viruses" of pleuropneumonia and agalactia requires special consideration. The causative agents pass readily through filters which retain bacteria but are cultivated with ease on liquid and solid media of a simple and lifeless constitution totally unsuited to viruses generally. The cell elements in both bear a close resemblance. Recently, Ledingham (1932) has succeeded in obtaining impression preparations of colonial growth at different stages, which, stained with Giemsa, reveal an extraordinary polymorphism. While no definite conclusions are as yet possible with regard to the precise systematic position of the organisms these preparations suggest affinities with the fungi or myxobacteria. The initial stages of growth would appear to be characterised by development of mycelial filaments from minute spores, and on this point the illustrated communications of Orskov (1927) and Wroblewski (1931) should be consulted. It is premature to discuss the possible relationship between such filterable bacterial forms and the elementary bodies in certain true viruses which have practically been established as the true virus agents. But for purposes of cultivation there would seem to be little to be learned from these organisms.

The legend, born of experience, that viruses are difficult to cultivate and in many cases apparently resistant to cultivation has reflected definitely on the acceptance of their living organismal nature. But with the realisation that certain virus bodies may now be obtained in a pure state improved technical methods of cultivation may clarify the true nature of the viruses.

VI. SUMMARY.

The two generally held theories of the nature of the filterable viruses affecting animals are outlined. The living, particulate nature of these is discussed in the light of present research and their almost certain affinities with the known bacteria. The weight of evidence is almost wholly in favour of this view.

The importance of cultivation as evidence in favour of the living nature of the viruses is pointed out and the cultivation of vaccinia virus discussed in some detail. A short survey of the cultivation of some other viruses is given mainly to show that the methods successful in vaccinia have proved successful with other viruses.

The requirements of filterable viruses for successful culture are discussed in so far as knowledge at present permits. The bulk of opinion is in favour of an intimate relation between living susceptible cells and the elementary bodies which constitute the virus. The possibility of culture in the absence of cells is dealt with and the evidence on this point outlined. The significance of the interrelation of elementary bodies, virus inclusion bodies and cultivation is indicated.

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THE INFLUENCE ON TISSUE PERMEABILITY OF A SUBSTANCE EXTRACTED FROM MAMMALIAN TESTES

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(With One Text-figure.)

CONTENTS.

I. INTRODUCTION.

DURING the past four years many papers have appeared in journals of experimental pathology in which the influence of an aqueous extract of mammalian testes on dermal infections with various viruses and bacteria have been described. The action on lesions produced by toxins and on the growth of tumour transplants have also been investigated. When this extract is added to the infecting agent and the mixture is inoculated intradermally the local lesion is markedly increased in size and altered in macroscopic appearance. The lesion is associated with an increased oedema of the surrounding skin and subcutaneous tissues, and, in some instances, the general invasive power of the infective or toxic agent is enhanced. Some of the workers described and investigated the direct effect of this extract on the dermis whereby the permeability of this tissue to injected material is greatly increased, and their experiments suggest that this alteration of permeability is responsible for the augmented size of the lesions. The enhanced permeability does not require delicate methods for its appreciation; it is an immediate and striking phenomenon that is easily visible to the naked eye.

The ability of testicular extract to increase the permeability of the dermis and other tissues is of considerable biological interest and it is for this reason that it has

seemed desirable to review in a biological journal the work which has been done on this subject. The pathological interest of the earlier observations has tended to obscure their biological significance and has limited the available articles almost entirely to journals of pathology.

II. INFLUENCE OF THE TESTICULAR EXTRACT ON VIRAL AND BACTERIAL INFECTIONS.

Duran-Reynals (1929) described the influence of extracts from certain mammalian tissues on the infecting power of vaccine virus. This paper followed his preliminary notes in 1928 which also described the influence of testicular extract upon the skin lesions produced by staphylococci. He found that aqueous extracts from testes of normal rabbits enhanced to an extraordinary degree the infectivity of strains of vaccinia and that extracts of some other organs, notably brain, liver and kidney, possessed this enhancing property, but in a much less degree than the testis. On the other hand, spleen, blood and bone marrow had the contrary effect and actually restrained or entirely suppressed the vaccinial skin reaction. The virus was not modified in its virulence by inoculation with the extract; strains obtained from the enhanced lesions were no more highly infective than those secured from an ordinary lesion. He found also that the augmenting substance was able to pass through a Berkefeld V candle and was carried down with the proteins when these were precipitated by weak acids.

Reynals found that the enhancing action was not species-specific, since extracts made from guinea-pig and rat testis caused the same effect in the rabbit skin. An area of skin when inoculated with testicular extract alone showed an enhanced lesion on the subsequent injection of virus, and the reaction was obtained as late as 3 days after the initial inoculation. He concluded from his experiments that the influence of the extract was on the cells of the host rather than on the virus, and suggested that the extract might cause increased cell division with resulting increased activity of the virus. In a private communication Duran-Reynals has since explained that these words did not express his meaning exactly, since no evidence of increased cell division was found in sections of skin. He wished to say that perhaps the extract created in the cell a state of susceptibility similar to that shown by dividing or young cells. Stewart and Duran-Reynals (1929) studied the influence of testicular extract upon the dissemination of the lesions of vaccinia virus throughout the body. The local lesion produced in the skin at the site of inoculation had been shown to be greatly increased in size by the addition of the extract, and they investigated the distribution and pathology of the lesions resulting from intracutaneous and intravenous inoculation of the virus together with testicular extract. They investigated the distribution and the pathology of the lesions caused by the generalised infection resulting from the intradermal inoculation of neurovirus together with testicular extract. Although testicular extract when injected intracutaneously with the virus had a marked enhancing effect, the same mixture when inoculated intravenously showed no sign of any such enhancement.

Hoffmann (1931) extended the observations on the action of the extract on vaccinia to three other virus diseases, namely herpes, vesicular stomatitis of horses and Borna disease. The lesions of these viruses were accentuated by the extract and a weak strain might be converted into a strong one. Pijoan (1931) investigated the action of the extract upon inoculations of twenty different types of bacteria and found that their infectivity was markedly enhanced. Kidney extracts enhanced the lesions of staphylococci to a slight degree, but spleen extracts never gave rise to enhancement and often inhibited the lesion. He does not mention the diffusion of the inoculation caused by the extract and considers that the enhanced effect might be due to increased cell multiplication or to alterations in the mechanism of injury and repair.

III. ACTION OF THE EXTRACT ON DERMAL PERMEABILITY AND PRELIMINARY OBSERVATIONS ON ITS SOURCE AND NATURE.

McClean (1930) investigated the influence of testicular extract on dermal permeability and the response to vaccine virus. He confirmed the enhancing effect of the extract on the dermal lesions described by Duran-Reynals, and, in addition, he found that the testicular extract alone produced a marked and immediate effect upon the dermis. An intracutaneous[1] injection of saline or phosphate solution produces a well-marked wheal, the margins of which remain quite distinct for 20–30 min. An injection of testicular extract, on the contrary, immediately diffuses into the dermis so that after 30 sec. to 1 min. it is difficult to identify the site of injection. This diffusion is so striking that it can be compared to the different behaviour of a drop of water when placed upon glazed paper and upon blotting paper. This effect had apparently not been observed by Duran-Reynals in his experiments already described, but was independently confirmed by Hoffmann and himself in a preliminary note (1930) and fully described in a later paper (1931). McClean found that the enhancement of the virus lesions was associated with this increased dermal permeability and was proportional to the amount of diffusion of the inoculum in the dermis. Experiments with Indian ink showed that these particles were spread over a larger area of the dermis by the influence of the extract (Fig. 1), and it seemed likely that the virus particles would be similarly dispersed. This dispersion would explain the increased virus response, and there is thus no need to postulate any increased cell division. The increased tissue permeability also explains the oedematous character of the augmented virus lesions. Intravenous inoculation of the extract enhanced the size of virus lesions produced locally by intradermal inoculation and the extract had a similar enhancing action on the dermal lesions of a typical bacterial toxin, that of *C. diphtheriae*. Extracts from other organs did not show visible increased diffusion in the skin, and, with the exception of kidney extract with which there was very slight enhancement, did not augment the activity of the virus.

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[1] By "intracutaneous" is meant an injection in which the needle is introduced through the Malpighian layer and the inoculation made so that the material lies in the dense connective tissue of the cutis vera beneath the Malpighian layer and superficial to the muscularis carnosus. This produces a small white wheal with its surface pitted by hair follicles and openings of the sweat ducts.

McClean (1930) reported preliminary experiments on the purification of the crude aqueous testis extract. He confirmed Duran-Reynals' report that the crude extract, which was active in dilutions from 1 : 500 to 1 : 1000, could be passed through a Berkefeld filter, without significant loss of activity, but he was unable to confirm
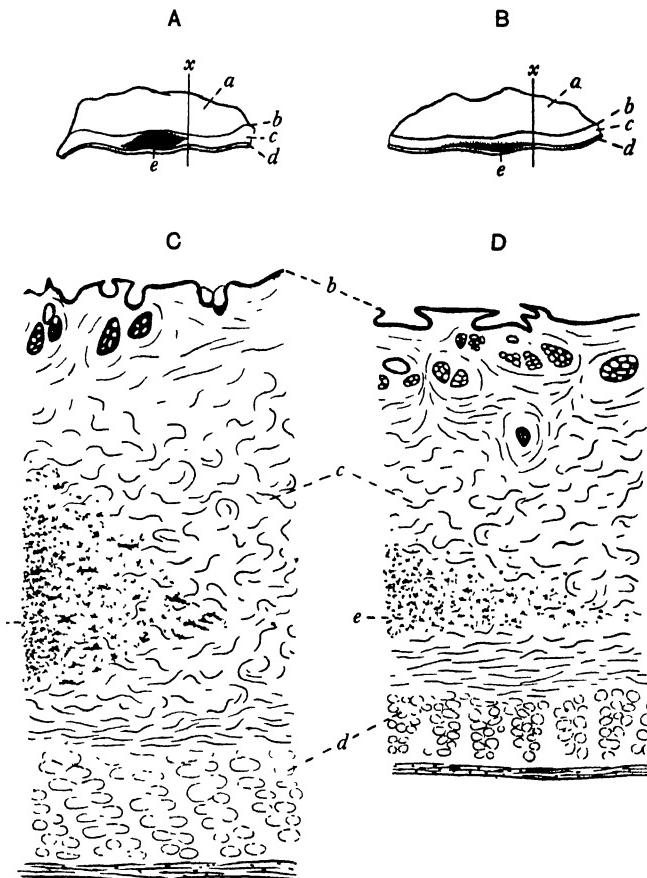

Fig. 1. A, inoculation of Indian ink with saline; naked-eye view of site of inoculation divided diametrically. B, inoculation of Indian ink with active fraction of testicular extract; view as A. C, drawing of section from specimen shown in A; the drawing was made at the point indicated by line *x*. D, drawing of section from specimen shown in B; the drawing was made at the point indicated by line *x*. *a*, surface of skin, seen in perspective; *b*, Malpighian layer; *c*, dermis; *d*, muscularis carnosus; *e*, Indian ink particles.

the statement that the active substance was precipitated by weak acids. He found that a small part of the active substance was adsorbed to the protein precipitate, but that most of it remained in the supernatant fluid. The precipitate obtained on the addition of a mixture of alcohol and ether contained the active substance which could be redissolved by suspending this precipitate in water. Preliminary qualita-

tive tests on this solution indicated that the active substance was not a carbohydrate and the tests for protein were doubtful.

It was thought that this substance might be a protamine or histone, since the former can be isolated in considerable quantities from the ripe sperm of fishes. Experiments with these substances yielded interesting results: it was found that protamine solutions produced a comparable diffusion in the dermis, but inhibited the virus lesions. The histone tested was without effect. Attempts to isolate a protamine from mammalian testes and to demonstrate a diffusion effect were unsuccessful.

McClean (1931) reported further observations on testis extract and its effect upon tissue permeability. His attempts to isolate the active substance were unsuccessful but some useful information was gained. The alcohol-ether precipitate already described or the dried minced tissue was extracted for 5 hours by boiling with chloroform or petroleum ether without inactivation; the active substance remained in the insoluble fraction and was obtained in an aqueous suspension of this fraction. The aqueous solution prepared from the dried alcohol-ether precipitate could be sterilised without loss of activity by saturation with chloroform, or the precipitate could be sterilised by dry heat at 160° C. for 30 min. It may be noted here that the active diffusing substance in testis extract is stable under ordinary conditions of storage provided that bacterial contamination is prevented and the reaction is kept within the limits of pH 5·0–8·5.

The permeability of the dermis was increased by the extract when the skin was removed from the killed animal and the extract was injected at intervals up to 48 hours after death. This indicated that the enhanced permeability was not due to altered vaso-motor or any other nervous response of the inoculated animal. Furthermore, skin which had been previously dried over sulphuric acid *in vacuo* and then soaked in water still showed increased permeability to the extract. Frozen sections of skin inoculated with extracts of testis, and also kidney, spermatozoa, a protamine solution, and, as controls, distilled water and saline, were examined for any visible effect on the structure of the dermis. The extracts of testis, spermatozoa and protamine solution produced marked "splitting" and swelling of the fibre bundles which were distorted so that the normal woven appearance of the dermis was destroyed. Kidney extract produced the same effect in a very slight degree. Saline and distilled water injected alone did not produce this effect. Indian ink particles injected at the same time could not be seen to have actually penetrated the fibrils, but this fact did not exclude the possibility that more finely divided material might do so. Hoffmann and Duran-Reynals (1931) used solutions of Prussian blue and methylene blue in addition to Indian ink. The Prussian blue was precipitated by fixing the tissues with formalin containing 5 per cent. hydrochloric acid. With this technique, they were able to show that the Prussian blue had penetrated within the cells of the connective tissue in the presence of testis extract to a much greater extent than when saline only was inoculated. Extracts of other organs and serum did not increase the spread of either Indian ink or dyes. They tested the action of injecting methylene blue intravenously and observing the effect on areas of skin previously inoculated with extract of testicle. They were of the opinion that there was greater penetration

of the dye in these areas than in those which had been inoculated with spleen extract or serum.

McClean (1931) found that there was no relation between the surface tension of solutions of testicular and other organ extracts and of protamines and their power to promote diffusion in the dermis. There was no relation between the diffusing power of these solutions on blotting paper and in the dermis, and, furthermore, testicular extract did not promote the diffusion of Indian ink, methylene blue or red blood corpuscles into gelatine or agar.

The production of spermatozoa is one of the most important functions of the testis, and it was therefore of interest to determine whether spermatozoa contain the active diffusing substance. The testis is peculiarly rich in this substance, and it seemed probable that this activity might be related to the primary function of the gland and not to a property of the interstitial tissue. Extracts of human, rabbit and guinea-pig spermatozoa were all found to be highly active, and in the case of human spermatozoa any chance contamination by prostatic fluid or secretion of Cowper's gland was eliminated by obtaining the spermatozoa from the contents of spermatocoeles removed at operation. Hoffman and Duran-Reynals (1931) also had observed that extracts of epididymis and rabbit sperm were highly active. They satisfied themselves that the active substance was not related to the male sex hormone. Reference will be made later in this article to further experimental work on the possible relation of this substance to the process of fertilisation.

Hoffmann and Duran-Reynals (1931) concluded that the extract caused no increase in the activity of toxins (tetanus and *B. coli*) or of trypsin. The negative results with trypsin may be due to the fact that this enzyme itself destroys the activity of the extract (McClean, 1931). The result obtained by Hoffmann and Duran-Reynals (1931) with tetanus toxin conflicts with that obtained by McClean when using diphtheria toxin. The dose of tetanus toxin was injected subcutaneously, whereas the diphtheria toxin was injected intracutaneously. Therefore, if the extract had any influence upon the absorption of tetanus toxin, similar to that which it has recently been shown to exert on the absorption of antitoxin (McClean and Morgan, 1933), it might be expected to have hastened its distribution throughout the body and thus reduced the incidence of local tetanus, an increase of which these authors were expecting. The augmenting effect of the extract on the skin lesion of diphtheria toxin is so constant that it has been used by Morgan and McClean as a routine test for the activity of different preparations in their purification experiments.

IV. INFLUENCE ON TUMOUR GROWTH.

In view of the enhancing effect of testis extract upon the lesions in the skin caused by bacteria and viruses it was of interest to observe the influence upon those tumours which can be propagated in animals, either by transplantation of the malignant cells or by cell-free filtrates. It was hoped that the observations might throw some light upon the nature of these carcinogenic agents. Since this work does not bear directly on the physiological activity of this extract, and since no very

conclusive or illuminating results have been obtained, it is not proposed to review these papers in any detail; Duran-Reynals (1930, 1931 *b*) described the influence of the extract upon the growth of a transplantable epithelial tumour of the rabbit. He found that, in contrast to the action upon viral and bacterial infections, the growth of this tumour was inhibited or completely suppressed. Hoffmann, Parkes and Walker (1931), on the other hand, working with the Rous sarcoma, which can be propagated with cell-free filtrates, obtained marked enhancement of tumour growth. Sturm and Duran-Reynals (1932) were unable to confirm these results; in their hands the extract showed no definite enhancing or inhibiting effect with this tumour. Tanzer (1932) investigated the influence on a variety of transplantable tumours of the mouse; some of these were inhibited by the extract. His experiments led him to suggest that the tumour-inhibiting and virus-enhancing substances in this extract were not identical. All the tumours used by Tanzer were propagated by transplants of tissue and not by cell-free filtrates. Duran-Reynals and Stewart (1931), approaching the problem from a different angle, investigated the action of tumour extracts upon the spread of vaccinia lesions and Indian ink particles.

The significance of these observations on the activity of tumours exposed to testis extract is not clear, and, moreover, the results of different workers are not in complete agreement. In so far as any general conclusion can be drawn, it seems that the extract exerts a definite inhibitory influence upon those tumours which are propagated by transplantation of cells, but that it may cause enhancement of tumours transmitted by cell-free filtrates. If this conclusion proves to be true and if the extract exerts its influence by increasing cell permeability, this result would not be surprising; it is natural to suppose that the tumour cells might be devitalised by contact with the extract while the cell-free filtrates, like the infective viruses, might have their activity enhanced by the increased permeability of the tissues into which they are injected.

V. OTHER SOURCES OF THE DIFFUSING SUBSTANCE.

Preliminary observations on the diffusing activity in the dermis of extracts from organs other than the testis have already been described. Recent work by Claude and Duran-Reynals (not yet published) has shown that extracts may be obtained from several organs, notably liver, kidney, lung, and spleen, which contain the active substance. It appears, however, from their experiments, that the yield of active material from these organs does not approach that obtained from the testis. These authors have found that the apparent inactivity of spleen extracts in their early experiments was due to the concentration of immune substances in this organ which inhibited the development of the viral lesions. Duran-Reynals has also extracted from the bodies of virulent bacteria a substance which increases tissue permeability; further developments of this work may be of great interest and may throw some light on the variable invasive power of different strains of bacteria. Spinelli (1932) has recently reported that the thyroid gland is rich in a substance which increases dermal permeability. I have confirmed this observation, but have found that the

activity of extracts of this gland is very small compared with those obtained from the testis. Hanger (1931) found that secretions from the upper respiratory tract enhanced the dermal lesions of *Bacillus lepisepticum* in the rabbit, and he considered the phenomenon to be analogous to that described by Duran-Reynals. It appears, however, that he was not aware at the time that extract of testis caused an immediate increase of dermal permeability and he did not test his material for this property. Therefore it is not clear whether the events described by him are related to the activity of testis extract.

VI. POSSIBLE ANTIGENIC CHARACTER OF THE EXTRACT.

Duran-Reynals (1932) carried out experiments to decide whether the diffusing factor in testis extract was antigenic in the strict sense of the term, that is to say, whether the active substance stimulated the production of antibodies in the circulation of animals inoculated parenterally with it. He immunised rabbits with both the crude aqueous extract and with a purified fraction which contained the active substance in concentrated form. Although some of his *in vitro* experiments suggested that the rabbit antisera might be exerting some neutralising action on the extract, all his results were complicated by the impossibility of distinguishing between a reaction due to general tissue immunity as opposed to a strictly specific reaction with the diffusing factor which is present in the extract. The *in vivo* experiments showed no neutralisation and supported the view that the *in vitro* results were due to an indirect reaction. Duran-Reynals' results indicated, but did not definitely prove, that the active factor did not itself stimulate the formation of antibodies in the sera of animals of the same or different species. Even if he had obtained positive results in his tests it is not clear that light would have been thrown on the nature of the diffusing factor; even the purified extracts with which he worked were not really pure and still contained an unspecified amount of inactive material. It is known that certain serologically specific substances, if linked to a protein, can provoke the production of antibodies though they are not themselves antigenic.

VII. INFLUENCE ON THE PERMEABILITY OF OTHER TISSUES.

Favilli (1931) published some interesting experiments on the action of the extract on the "fragility" of red blood cells. He found that contact with the extract *in vitro* caused a marked alteration in the sensitivity of red cells to dilution of the salt content of saline in which they were suspended. Favilli worked with extract from the testes of rats, rabbits, and guinea-pigs and tested all of them against the red cells of each of the three species. He found that whereas rat extract was the most and guinea-pig extract the least active in the dermis, the converse was true of the relative sensitivity of the red cells; in other words, the species with the most active testicular extract had the least sensitive cells. He found that the fragility of human red cells was markedly increased by rat testis extract, and that a purified extract from bull's testis affected the red cells of the rat and rabbit in the same manner. No *in vivo* action of the extract on the red cells could be demonstrated, guinea-pigs which were

intravenously injected with rat testis extract showed no change in the fragility of their cells. Favilli observed the action of spleen extracts from the same species on the red blood cells and found that the spleen extracts very slightly increased their fragility, but not to a degree in any way comparable with extract of testis.

Favilli's results not only support the hypothesis that the factor in the testis extract which enhances infections does so by increasing the permeability of the tissues, but, in addition, they show that this effect on permeability is not confined to the dermis. These observations have been confirmed by Gaetani (1932).

McClean and Morgan (1933) investigated the influence of the extract on the absorption of diphtheria antitoxin in the guinea-pig. A standard dose of antitoxin was injected subcutaneously and intramuscularly with and without the addition of extract. Samples of blood were withdrawn from both groups of animals at regular intervals after this inoculation. When the antitoxin was administered subcutaneously there was a 100 per cent. increase of absorption of antitoxin at 2 and 6 hours in those animals which had received extract; at 24 hours the difference, though less marked, was still definite. Intramuscular inoculation showed no significant increase in the rate of absorption of antitoxin. This action of the extract on the absorption of a substance inoculated subcutaneously confirmed the conclusion drawn from Favilli's experiments that the enhancement of tissue permeability is not confined to the dermis.

VIII. ACTION ON ECHINODERM EGGS.

It is known that there is a marked increase in the permeability of eggs immediately after fertilisation. A considerable amount of experimental work has been undertaken to elucidate the nature of this change, which is thought to be a surface effect (Carter, 1924) and is apparently initiated by the entry of the sperm. The testis is peculiarly rich in the diffusing substance which, moreover, can be extracted from spermatozoa, and it seemed that the power of these extracts to increase tissue permeability might be related to the function of the spermatozoa. Some preliminary observations reported by McClean (1931) suggested that further work might be fruitful, and experiments performed by Hobson and McClean at the Marine Biological Laboratory, Plymouth, in 1931 were designed to throw further light on this aspect of the problem. Hobson had been working on permeability changes in eggs, and the technique employed is fully described in his papers (1932). Since the results obtained with this extract by these workers were inconclusive, and since it is hoped to repeat and extend this work as soon as possible, these experiments have not hitherto been published, but in view of the observations reported by Favilli (1932), a brief summary of the results obtained may be given here.

Two samples of extract of mammalian testis were used in all these experiments; a solution obtained from the alcohol-ether precipitate already referred to, and a solution obtained by a method of purification to be described later (Morgan and McClean, 1932). These extracts were active in the dermis at dilution of 1:250 and 1:1000 respectively. In addition, in one experiment a crude extract of sperm from *Psammechinus miliaris* was used, and in another, a sample of the purified fraction

which had been employed by Duran-Reynals and his co-workers. After experimental treatment with .the various solutions serial photographs of groups of 60–80 eggs were taken, and these were either measured for relative swelling rates (Hobson, 1932) or examined for percentages of ova showing cleavage.

After *Psammechinus miliaris* eggs had been exposed to the various samples of extract diluted in sea water, they were either immersed in 50 per cent. sea water and the swelling rate compared with that of untreated control eggs, or they were inseminated and the percentage showing cleavage was compared with control eggs inseminated at the same time. In one experiment a crude aqueous extract of *P. miliaris* sperm was used in view of the possibility that a mammalian extract might not be suitable for an experiment with echinoids. It appeared, however, from these experiments that mammalian extracts were not toxic to these eggs, since after treatment they could be successfully fertilised and free swimming plutei obtained. Repeated experiments under varying experimental conditions failed to show any significant or constant influence of the extract either on the swelling rates or percentage of cleavages obtained. Similar experiments with unfertilised eggs of *Holothuria nigra* also gave negative results.

The influence of the extract on the permeability of *P. miliaris* eggs to acid and alkali was also investigated. Solutions of neutral red were used as indicator, and the eggs were treated with butyric acid and ammonium hydroxide in one experiment and with hydrochloric acid and sodium hydroxide in another. No action of the extract could be detected on the time taken for the colour change to occur or on the intensity of staining.

Favilli (1932) carried out similar experiments with the eggs of *Arbacia punctulata*. He used a screw micrometer and calculated his results from the formula devised by Lucké, Hartline and McCutcheon (1931), the volume being calculated from the diameter measurements. The extract was prepared from the testes of rats and guinea-pigs and in some experiments the purified fraction used by Duran-Reynals was employed. Parallel experiments were made using spleen extract instead of testis extract. In each experiment the normal size was based on the measurement of forty eggs, and in each test measurements of an average of six eggs were taken.

All Favilli's experiments gave consistent results and he found that contact with both testis and spleen extract greatly increased the swelling rate of the eggs. Using the formula referred to above, the permeability value obtained for the test eggs was 0·088 at the second minute and 0·092 at the third as compared with 0·036 and 0·050 respectively for the control eggs in sea water alone.

There was no significant difference between the action of spleen and testis extract. Purified testis extract, though less effective than the crude fresh extract, gave similar results. Blood serum in a dilution of 1:25 produced marked swelling, but in higher dilutions, comparable with those in which testis and spleen extracts were used, it was inactive. Other proteins, egg albumen, gelatine, and peptone were inactive. The organ extracts exerted no harmful action on the eggs, which after exposure to them and to diluted sea water were returned to normal sea water and successfully fertilised.

It is difficult to trace the cause of the discrepancy between the results of Favilli on the one hand and Hobson and McClean on the other. The technique employed by the latter workers was adequate to detect a difference of the order described by Favilli. The results obtained by Favilli with spleen extract and normal serum indicate the care with which all experiments should be controlled before a permeability change can be definitely ascribed to the specific action of the diffusing substance present in testis extract. In forming a conclusion as to the physiological significance of this diffusing substance in testis extract, these experiments on eggs are of cardinal importance, and it is essential that the results obtained by Favilli should be confirmed and extended.

IX. PURIFICATION OF THE ACTIVE SUBSTANCE.

The isolation of a biological substance capable of immediately and profoundly altering tissue permeability would be of considerable interest. Some preliminary observations by McClean upon the purification of the crude aqueous extracts of testis and upon the properties of the purified fraction have already been described. In several of the papers published by Duran-Reynals and his co-workers reference has been made to a purified extract with which experiments have been performed, and a paper by these authors giving full details of their methods and results, is in preparation. Meanwhile Duran-Reynals (1931 *b*) has published a review of the work on testicular extract in which a summary of the methods of purification employed by Claude, Helmer and himself is given. Their method depended upon the precipitation of the active substance with acetone from solution which had been purified with acetic acid and with ammonia. From 100 gm. of fresh testis they obtained 0·22 gm. of precipitate which apparently contained all the active substance, but no precise figures are given showing the relation between dry weight of solid matter and activity of the purified solutions, nor are there any reports of quantitative analyses of the purified material. There was some evidence that this active substance is not carbohydrate or protein in nature.

Morgan and McClean (1932) described a method of purification of the extract which depended upon the precipitation of the active substance as a basic lead salt and its subsequent fractionation with ammonium sulphate followed by ethyl alcohol. The minimal dose of this active fraction which would produce increased diffusion in the dermis was 0·0000010 mg., and solutions were obtained which were active in a dilution of 1 : 100,000. The fact that the active substance can be obtained in a heat-stable form indicates that it is not an enzyme. Using the same method of purification, Morgan and McClean were able to separate the active substance from the protamine sample which had shown active diffusion in the skin (McClean, 1930). It appeared that the apparent activity of the protamine salt was due to contamination with traces of the active substance.

X. SUMMARY.

A substance has been extracted from mammalian testes which produces a marked and immediate increase in the permeability of the dermis to injected material. This substance causes a marked enhancement in the size of dermal lesions produced by the injection of bacteria, viruses and toxins, and it does so by increasing the area of the skin over which these infective agents are dispersed. Microscopic examination of sections of skin injected with extracts of testis show definite changes in the appearance of the collagen fibres of the dermis.

Extracts have been obtained from other organs which cause increased diffusion in the skin, but the activity does not approach that of testicular extracts. - Spermatozoa are rich in this diffusing substance, and it is suggested that this substance is concerned in fertilisation. Experiments by different workers with the action of the extract on the permeability of echinoderm eggs have given conflicting results, and it is not yet clear whether the permeability of these eggs is increased.

The influence of this extract on tissue permeability is not confined to the dermis. It has been shown to increase the "fragility" of red blood cells and to increase the absorption of antitoxin administered subcutaneously. There does not appear to be any action on the permeability of voluntary muscle.

Methods of purification of the extracts have been described which have resulted in the separation of highly active fractions. Definite increased dermal diffusion is associated with 0·0000010 mg. dry weight of solid matter. The active substance is insoluble in the usual fat solvents, and preliminary chemical tests indicate that it is neither carbohydrate nor protein in nature.

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VITAL STAINING IN RELATION TO CELL PHYSIOLOGY AND PATHOLOGY

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(With Two Text-figures.)

CONTENTS.

I. INTRODUCTION.

VITAL staining embraces a variety of methods employed for the study of the structure and function of cells, as well as for the identification of specific types of cells. It is not intended here to make a comprehensive review of the extensive literature of the subject, but rather to direct attention to certain aspects of recent work which have opened up wide fields for further investigation.

A cell is said to be vitally stained when it acquires coloration, while still alive, as the result of dyestuff being applied to it. There are various types of vital staining, which involve different intracellular processes. There is the staining of preformed cell structures, such as mitochondria, cytoplasmic granules, and vacuoles. There is the diffuse coloration of living protoplasm. There is also the formation of dye droplets in the cytoplasm by the process of segregation ("Speicherung"). In reviews of this subject von Möllendorff (1920, 1921, 1926) has expressed the opinion that in general acid dyestuffs are segregated, while basic dyes either stain preformed cytoplasmic inclusions or colour protoplasm diffusely.

II. VITAL STAINS AND METHODS OF VITAL STAINING.

Almost all the dyes which have been employed for vital staining are included in one or other of the classes of acid or basic dyes. Acid dyes are salts of a colour acid with a base, most frequently Na, K or Ca; while basic dyes are salts of a colour base with an acid radicle, usually $-Cl$ or $-SO_4$. In the following list the dyes to which

reference is made in this article are arranged according to their chemical constitution:

ACID DYES.

Azo-dyes.

Orange G   Trypan blue.
Isamine blue   Vital new red.

Fluorane derivatives.

Eosin

BASIC DYES.

Azines.

Neutral red   Janus green (also azo-chromaphore).

Triamino-tri-phenyl-methanes.

Methyl violet   Gentian violet.
Dahlia

Thiazines.

Methylene blue   Toluidine blue.

Oxazines.

Brilliant cresyl blue.

Rhodamines.

Rhodamine B.

Detailed information as to the chemical constitution, methods of preparation, etc. of dyestuffs are given in the Colour Index (Society of Dyers and Colourists, Bradford, 1924. Supplement, 1928). Constitutional formulæ for a large number of acid dyes are also given by Schulemann (1917).

The methods employed for vital staining depend upon the nature of the investigation and the organism chosen for the purpose. Small aquatic animals are invariably stained by the addition of dyestuffs to the water in which they are kept. Vital staining of terrestrial animals is usually accomplished by injecting dilute solutions of the more diffusible dyes, either subcutaneously or intraperitoneally, or by injecting the less diffusible dyes intravenously. Basic dyes have also been injected subcutaneously and intraperitoneally for staining liver, kidney and pancreas cells. Staining of living animals by these methods is described as *intra vitam* staining or staining *in vivo*.

For vital staining with basic dyes the method most frequently employed has been the supra-vital technique, that is, fragments of living tissues are treated with dilute solutions of the dyes usually in physiological saline. The tissues may be teased out in the dye solution on a glass slide and examined immediately (on a warm stage in the case of homoiothermal animals), or may first be incubated in the dye solution at the body temperature of the organism.

One of the best methods for observing vital staining is to employ cells growing in tissue cultures. The dye is either added to the culture medium at the time of explantation, or introduced into the culture when growth has commenced. This method is referred to as vital staining *in vitro*. With the tissues of homoiothermal animals observations can be carried out over prolonged periods by having the microscope in a special box, electrically controlled at the body temperature of the animal whose tissues are being examined. For some purposes it is an advantage to have the culture, while on the stage of the microscope, connected up with suitable irrigation apparatus so that the dye solutions can be allowed to flow over the cells and be washed away again without interrupting microscopical examination. By using such apparatus, cells can be subjected to the action of various reagents before application of the dyes, and thus the influence on vital staining of substances with different physiological properties can be directly observed.

III. SEGREGATION OF ACID DYES.

One of the most convenient ways of studying segregation is by introducing a drop of dilute trypan blue solution into a tissue culture of living fibroblasts. Within 24–48 hours blue droplets make their appearance in the cells (Fig. 1 A). It is generally agreed that these are new formations induced by the presence of the dye in the cytoplasm. The dye is separated from the living protoplasm by virtue of a segregating power of the cell; and for such segregation ("Speicherung") minute vesicles and vacuoles are formed. There is thus brought about "an actual accumulation within the cell of vital dyestuffs in fluid, high-colloidal, flocculated, or crystalline form" (Evans and Scott, 1921). According to von Möllendorff (1921) "die Entstehung des Färbungsbildes beweist das die Granulabildung auf rein physikalische Vorgänge zurückzuführen ist." Chlopin (1930) has shown in the case of sugar of iron, the segregation of which takes place in the same manner as trypan blue, that there is a protein substance associated with the dye droplets, at least in advanced segregation. After removal of the iron from the cells, the protein particles remain and can be stained with acid dyes.

For the purpose of ascertaining upon what properties of dyestuffs their capacity for vital staining depends, Schulemann (1917) carried out experiments *in vivo* with a large number of acid dyes. He came to the conclusion that their rate of diffusion was the determining factor, a result with which von Möllendorff is in agreement. It is maintained that highly diffusible dyes spread rapidly through the animal body and are rapidly excreted. Indiffusible dyes and colloids when injected subcutaneously are deposited at the site of injection. It is the dyestuffs with medium rates of diffusion, the so-called semi-colloids, which are the best vital dyes. It has thus been concluded that chemical constitution has no direct relation to vital staining, but that factors which influence rate of diffusion are effective.

In the parenchyma cells of the liver of the mouse Ludford (1931) found that the highly diffusible acid dyes eosin and orange G, when injected subcutaneously or interperitoneally, were not segregated, while the less diffusible acid dyes trypan blue

and vital new red were. The dyestuff which was segregated as droplets in these cells was believed to have followed the normal path of secretion in the liver cell. Evidence was adduced that this process is dependent upon the state of functional activity of the cells.

Wallbach (1931 *a*) investigated the action of twenty-two acid dyes upon fibroblasts and macrophages in tissue cultures of the rabbit spleen. He found no relation between the rates of diffusion of these dyes and their vital staining properties (segregation), and he was led to conclude, "dass jeder vitale Farbstoff zu seiner
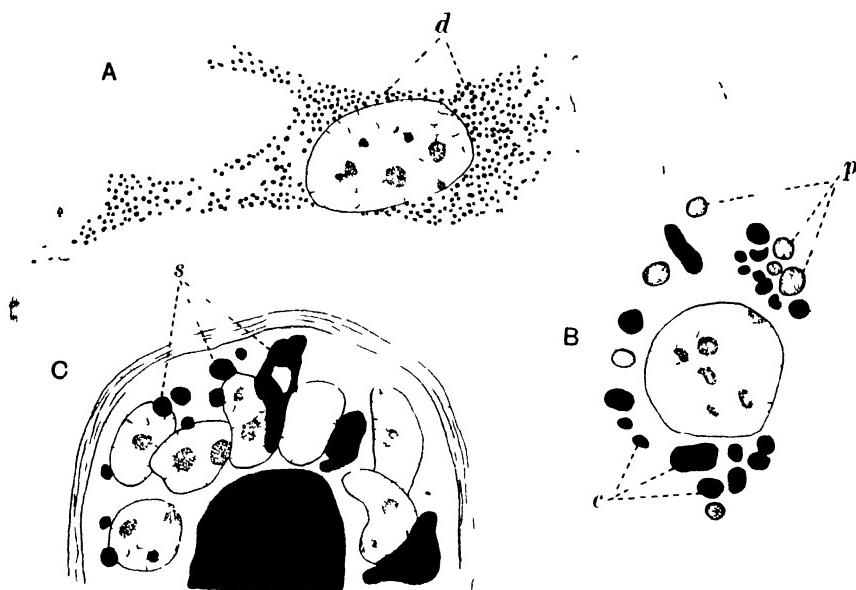

Fig 1 Types of vital staining with the acid dye trypan blue A, intense segregation of the dye in droplet form (*d*) in a fibroblast from a tissue culture of mouse embryo heart B, phagocytosis of coloured (*c*) and uncoloured (*p*) particles in a malignant cell of the Crocker sarcoma No segregation has occurred in this cell C, staining of the secretion (*s*) in mammary gland cells of the mouse The milk in the lumen of the acinus is also stained (B and C after Ludford, 1932, more diagrammatic than the original figures)

Aufnahme eines besonderen funktionellen Zustandes der Zellen bedarf." Wallbach considers that the chemical constitution of dyestuffs plays an important part in determining whether they will be segregated by cells. Cappell (1929) had previously expressed similar views.

Clearly the whole question of the vital staining properties of acid dyestuffs needs reinvestigating. We need to know to what extent the physical properties of dyestuffs influence segregation *in vivo* and *in vitro*, whether segregation is determined by the same factors in different types of cells, and upon what chemical properties of the dyestuffs segregation may depend.

A striking example of a change in functional activity of cells being accompanied by an altered reaction to an acid dye is afforded by malignant cells. Thus Ludford (1929, 1932) has shown that both *in vivo* and *in vitro* malignant cells of the mouse fail to segregate trypan blue in the same manner as their non-malignant prototypes. Similar differences were observed between normal cells and the cells of filterable tumours of the fowl (see also Foulds (1932)). The reason for this difference in the reaction of normal and of malignant cells to trypan blue is not clear. It may be the result of the peculiar metabolism of the malignant cell, or be due to some difference in the colloidal state of the protoplasm. There is also the possibility that malignant cells are less permeable to trypan blue than their non-malignant prototypes.

Some investigators have held the view that the taking up of semi-colloid acid dyes by cells is a similar process to the phagocytosis of particulate matter. The study of vitally stained tissue cultures of tumours has shown that this conception cannot be correct. Thus Ludford (1932) has described the phagocytosis of coloured *débris* by malignant cells which have failed to segregate the colouring matter, in this case trypan blue (Fig. 1 B). The manner in which acid dyes penetrate living cells merits further consideration.

IV. STAINING OF CELL GRANULES AND VACUOLES.

There has been considerable controversy as to the nature of the cell granules stainable by basic dyes such as neutral red. They have been regarded as part of the living structure of the cell, as metaplastic bodies, as vacuoles resulting from changes induced in the colloidal state of the protoplasm, and as products of the segregative activity of the cells. There is little doubt that different investigators have been concerned with different things; but granules in one and the same type of cell have been differently interpreted.

It is clearly established that there are a number of basic dyes which stain non-living cell inclusions, such as cytoplasmic vacuoles, phagocytosed bodies, yolk granules of embryonic tissues and dye droplets formed in cells as the result of vital staining with acid dyes. The results of supra-vital staining tests carried out with a number of basic dyes led von Möllendorff (1920) to conclude that the best basic dyes for staining cell inclusions were those which were readily flocculated in contact with acid colloids, but were only slightly soluble in lipoids.

The staining of preformed cell structures is not the unique property of basic dyes. Acid dyes colour protein granules in epithelial cells of the intestine of young mice, the secretion in mammary gland cells (Fig. 1 C), and in certain of the ductless glands (von Möllendorff, 1921), and granules regarded as keratohyalin in epidermal cells of tissue cultures of embryo skin (Ludford, 1932). In invertebrate cells also Chlopin (1927) has described the staining of preformed inclusions.

The fact that both acid and basic dyes stain non-living substances in cells suggests the possibility that the chemical constitution of the dyestuff, as well as that of the stainable material, may play an important part in this type of staining, but this is still an unexplored topic.

The relation between the state of functional activity of a cell and the staining of its cytoplasmic granules and vacuoles has not until recently been the subject of experimental enquiry. Hirsch (1931 a, 1931 b) has found that the *intra vitam* staining of vacuoles in acinar cells of the pancreas with neutral red is influenced by the permeability of the cells, which varies at different phases of the secretory cycle. Nagel (1929, 1930, 1931) has found that the staining of fibroblasts with neutral red and methylene blue *in vitro* is influenced by the condition of the cultures, the temperature, and the intensity of illumination. Further he found that vital staining with methylene blue is accelerated by the narcotics methyl and ethyl alcohols and ethyl urethane. This result he attributes to their retarding influence on cell metabolism. He believes that the methylene blue which enters the cells, being less affected by oxidation-reduction processes, is deposited in larger amounts and from greater dilutions.

Differences in the vital staining of different types of cells have been clearly demonstrated by Gicklhorn (1931). He has shown that with some dyes under certain conditions it is possible to colour individual organs, or even specific tissues, while the rest of the organism remains uncoloured. This type of staining he calls "Elektive Vitalfärbung." Leber (1932) found that with a mixture of neutral red and methylene blue in definite dilutions it is possible to stain differently the two types of cells of the "epipodialen Anhänge (Kiemen oder Kiemensäckchen)" of *Daphnia*, so that one stains with neutral red, and the other with methylene blue. Such results afford clear evidence that in granule staining with basic dyes the nature of the cell cannot be a negligible factor.

This type of investigation has shown a new way of approaching the study of cell physiology, by indicating a method for the morphological study of cellular activity.

V. SEGREGATION OF BASIC DYES.

Is there a fundamental difference between vital staining with acid and basic dyes, or are both alike segregated under appropriate conditions? Researches carried out within recent years afford evidence for the framing of a definite reply to these questions.

Chlopin (1927) investigated the vital staining with neutral red of a wide range of cells from invertebrates and lower vertebrates. He found that this dye not only stained preformed cytoplasmic granules and droplets, but also gave rise to new formations. With intense staining the newly formed dye droplets contained, almost without exception, varying amounts of a substance regarded as protein from the way in which it was retained after fixation, which in fixed tissues was stainable with basic aniline dyes. This substance he called the "Krinom." Its formation was not accompanied by any marked disturbance of the normal physiological function of the cells, or by any demonstrable pathological deformation of their morphology. Krjukowa (1929) investigated the vital staining of the salivary gland cells of the larva of *Chironomus* which led him also to conclude that neutral red gives rise to new cytoplasmic inclusions. Alexenko (1929, 1930), working with neurones of the fowl at

various stages of development, and Weiner (1930), studying oocytes of earthworms, both employing the supra-vital technique, came to similar conclusions, as did also Ludford (1930) from a study of acinar cells of the pancreas of the mouse stained *intra vitam*. Ludford also demonstrated in the same cells deposits associated with the dye droplets, which Chlopin calls the "Krinom" (Fig. 2 D), but he did not find any granule staining or segregation with the diffuse-staining dye rhodamine B. In liver cells it was demonstrated (Ludford, 1931) that both acid and basic dyes when injected into the living animal are segregated in the same manner in the same region of the cells, next to the intercellular bile canaliculi. Thus the acid dyes trypan blue and vital new red gave rise to coloured droplets, as did also the basic dyes neutral red, cresyl blue and toluidine blue, but the more lipoid-soluble basic dyes rhodamine B and methyl violet 6 B were not segregated. The "Krinom" formed after staining with neutral red was clearly demonstrable. Schlottke (1932), from his work on *Hydra*, has also concluded that in this organism there is no fundamental distinction between vital staining with acid and basic dyes.

An insight into the mechanism of segregation of basic dyes is afforded by the work of Nassonov (1930, 1932), who demonstrated that the epithelial cells of the frog's intestine segregate basic dyes under aerobic conditions, but that in an atmosphere of hydrogen no segregation takes place. Instead, the nucleus stains as the result of a gelation, which within limits is reversible, so that on the introduction of oxygen the colour disappears from the nucleus and dye droplets appear in the cytoplasm. Further experiments indicated that the nuclear gelation is the result of displacement of the pH of the protoplasm to the acid side. In these investigations the frog's gut was filled with the dye solution by a special technique (see Nassonov, 1930).

Basic dyes like neutral red would seem to stain all types of cells vitally. In some this is clearly a coloration of preformed inclusions, in others segregation such as occurs with acid dyes, and in some cells both processes occur. It would seem that a basic dye such as neutral red on entering a cell is flocculated at the surface of any droplets and granules which may be present, possibly only if they are of a more acid character. Excess of dye then becomes deposited as new cytoplasmic formations. Whether this applies to every cell remains to be determined.

VI. VITAL STAINING OF CELL ORGANS.

The specific staining of mitochondria with Janus green constitutes a special case of vital staining. The dye necessary for this purpose is diethylsafraninazodimethylanilin chloride, and according to Cowdry (1916) its action depends upon the diethylsafranin group since this alone will stain mitochondria more or less specifically. Other dyes which contain the same group and have similar staining reactions are Janus blue, Janus black, and Janus grey.

To what extent the staining of mitochondria with Janus green depends upon the state of the cells remains uncertain. Cowdry (1914) observed neutrophile leucocytes with vitally stained mitochondria performing amoeboid movements and phagocy-

tosing foreign particles. Other investigators believe that the staining does not occur in actively growing cells without interference with their metabolism. Cowdry (1926) found that the mitochondria of human lymphocytes stain with a dilution of Janus green in physiological saline of 1 : 500,000. "This means," he says, "that the dye, coming in contact with the mitochondria, is concentrated by them to a point which permits of visibility in a film about 0·5 micron in thickness, that is to say we are dealing with a concentration of several thousand times" (Cowdry, 1926). We are still without a satisfactory explanation of this remarkable example of "elective" vital staining.

Other basic dyes, *e.g.* dahlia and gentian violet, have been said to stain mitochondria in some cells but they are not of general application.

Cytologists have long sought for a specific stain for the Golgi bodies, or Golgi material as Bowen (1926) preferred to call it. These bodies are visible in the living cells of some invertebrates. They have been vitally stained with basic dyes in the male germ cells of pulmonate molluscs; but in most cases where they are visible in the living cell it has been impossible to stain them vitally. The literature of this subject has been reviewed by Bowen (1928). We are still seeking a dye which will stain the Golgi bodies specifically as Janus green does the mitochondria.

There is a general consensus of opinion that the nucleus of the animal cell cannot be vitally stained under normal condition of metabolic activity. However, Russell (1914) has described the growth of embryonic and adult tissues of the frog in plasma coloured by gentian violet in dilutions of from 1 : 4000 to 1 : 20,000. This dye, he claims, is a true nuclear stain and colours the chromosomes during cell division. I have been unable to repeat this result with mammalian cells.

Reference has already been made to the work of Nassonov (1930, 1932) who has described the vital staining of the nucleus in epithelial cells of the intestine of the frog under altered conditions of metabolism. It remains for further investigation to determine whether these results are applicable to the cells of homoiothermal animals.

VII. RÔLE OF THE CELL ORGANS IN VITAL STAINING.

Jasswoin (1925) was the first to point out that trypan blue droplets are formed in kidney cells in that area of the cytoplasm where the Golgi apparatus can be demonstrated by silver and osmic acid impregnation methods. Nassonov (1926) investigated the relationship between trypan blue droplets and the Golgi apparatus in the kidney and liver cells of several vertebrates and discovered complete agreement between the position and arrangement of dye droplets and of the Golgi apparatus in the early stages of *intra vitam* staining. Similar observations were recorded by Makarov (1926). While Glasunow (1928) described, after intense *intra vitam* staining with trypan blue, the anastomosis of dye droplets to form first filaments, and finally the typical reticulate form of the Golgi apparatus. Ludford (1928) was able to demonstrate in kidney and liver cells of the mouse, which had been injected either subcutaneously or intraperitoneally with trypan blue, both the Golgi apparatus

and dye droplets in intimate relationship with it. Dye droplets appeared to be formed in association with the Golgi apparatus in the same manner as secretion granules arise in gland cells. That is, the Golgi apparatus appears to bring about a concentration into droplets of both secretions and dyestuffs as Nassonov (1926) first pointed out. According to Ludford (1931) this applies to both acid and basic dyes in parenchyma cells of the liver.

A topographical relationship between the Golgi apparatus and the first formed droplets to appear in vital staining has been described in many other cells, *e.g.* in epidermal cells and in cells of the monocyte-macrophage series of the mouse (Ludford, 1929), in epithelial cells of the frog's intestine (Nassonov, 1930), and in several other types of gland cells.

The identical position in cells of red vacuoles, following vital staining with neutral red, and of the Golgi apparatus led Parat (1928) to formulate his "vacuome" theory according to which there are two structural elements universally present in cells, namely mitochondria and the vacuome, composed of vacuoles stainable with certain basic dyes, especially neutral red. Later he modified this theory and postulated the existence in the cytoplasm of a "zone of Golgi," which consisted of the "vacuome" in association with specialised mitochondria, which he calls the "*chondriome actif*" or dictyosomes. The latter are applied to the surface of the "vacuome," and around this area of the cytoplasm lipins accumulate owing to their property of lowering surface tension. A dictyosome applied to the surface of a vacuole constitutes an oil-water interface system. Here molecules of amino acids become orientated, and their condensation thus brought about facilitates the synthesis of proteins. The elaborated products collect in the vacuoles, condense into granules, and in this way secretion granules originate in gland cells. According to this theory the "zone of Golgi" is a specialised area of the cytoplasm particularly concerned with synthetic processes (Parat, 1928). More recently it has been suggested by Koehring (1930, 1931) and Kedrowsky (1932) that neutral red staining denotes the presence of enzymes, and that the staining of the vacuome with this dye indicates its enzymatic nature. The reticulate form of the Golgi apparatus demonstrable by osmic and silver impregnation techniques is regarded as an artifact resulting from faulty impregnation of the dictyosomes, or of dictyosomes and vacuome. This conception of cytoplasmic organisation has been supported by numerous papers from Parat's laboratory, and amongst others by Covell and Scott (1928) on the basis of their work on the vital staining of neurones of the mouse, and by Zweibaum and Elkner (1930) from their study of the vital staining of cells in tissue cultures.

This theory has, however, been subjected to much criticism. In a series of papers by Gatenby (1929), by Hirschler (1928), and by other workers from their laboratories, it has been shown that in the germ cells of a large number of different organisms vacuoles stainable with neutral red are present in addition to the Golgi bodies, which are often visible in the living cells. In acinar cells of the pancreas of the rat Beams (1930) found "the vacuome and the Golgi apparatus side by side in the same cell." Ludford (1930) showed that in intense *intra vitam* staining with neutral red, dye droplets originate in the region of the Golgi apparatus within acinar

cells of the mouse pancreas (Fig. 2 A, B). The formation of large dye droplets
resulted in the break-up of the reticulate form of the Golgi apparatus (Fig. 2 C).
The "Krinom" was also demonstrable in these cells (Fig. 2 D). Gatenby (1931)
believes that the fine droplets which he stained in pancreas cells of *Pseudotriton*
represent the prozymogen vacuoles of Bensley (1911).

Reference has already been made to the work of other investigators who have
shown that neutral red is segregated in cells like acid dyes.

It appears to me that the Golgi apparatus represents, at least in cells of a secre-
tory or excretory function, a specialised area of the cytoplasm where substances
elaborated by the cell, or prepared by it for elimination, are segregated into droplets
or granules. When such a cell is vitally stained with a dye like neutral red, if there
are any such droplets present, especially if they are of an acid character, they are
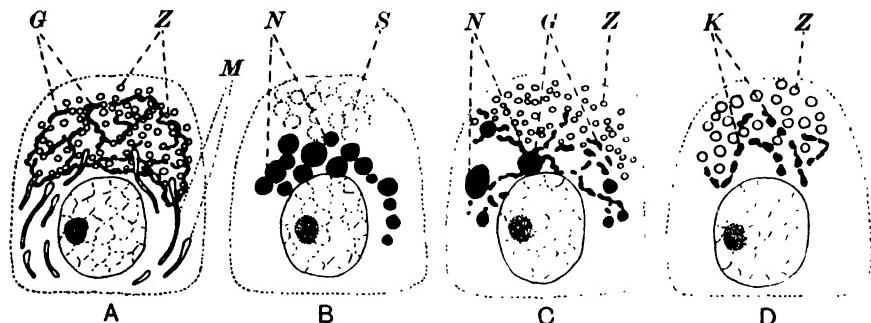
Fig. 2. Vital staining of acinar cells of the pancreas with neutral red. *G*, Golgi apparatus;
K, "Krinom"; *M*, mitochondria; *N*, dye droplets; *S*, secretion droplet; *Z*, zymogen granules.

A, cells not vitally stained showing the relationship between zymogen granules and the Golgi
apparatus. B, cell vitally stained with neutral red. C, breaking up of the Golgi apparatus in a cell
vitally stained with neutral red. D, the "Krinom" formed after vital staining with neutral red.
(Diagrams after Ludford, 1930.)

stained first by the flocculation of the dye. Excess dye may then be segregated in the
same manner as the specific secretions or excretions. There is no evidence that the
mitochondria play any direct part in this process (Nassonov, 1926; Ludford, 1928).

It remains to be determined whether the same mechanism is active in all cells.
The staining of vacuoles with neutral red in degenerating cells is probably quite a
different process. Vacuoles then appear to arise from phase separation of the proto-
plasmic colloids, and these readily flocculate basic dyes. Whether the vacuoles which
originate during the normal course of segregation of dyestuffs or secretion also
originate by the separation of phases of the cell colloids, is uncertain.

VIII. DIFFUSE STAINING OF CELLS.

There is a class of basic dyestuffs which stain living cells diffusely. According to
von Möllendorff (1920) they are readily soluble in lipins and show no disposition to
be flocculated by acid colloids. Some, such as rhodamine B, have not been found
either to stain preformed cytoplasmic inclusions or to be segregated (Ludford, 1931).

There are others, such as gentian violet, which are flocculated to a slight extent, giving a faint granular staining, and which at the same time stain protoplasm diffusely. Although some investigators have expressed the view that these dyestuffs are the most toxic, yet their influence upon cellular activity has not yet been adequately investigated.

Degenerating cells on reaching a certain state of disorganisation stain diffusely with acid dyes such as trypan blue. This is clearly demonstrable in tissue cultures of mouse sarcomata (Ludford, 1932). As has already been mentioned, the sarcoma cells do not segregate trypan blue, but in old cultures degenerating cells which, up to a certain time have remained colourless, begin to acquire coloration. The whole cell is ultimately stained, the nucleolar staining being usually particularly intense.

According to Wallbach (1931 *b*) not all dead cells stain diffusely. Such staining only occurs with certain types of necrosis, and is not necessarily a criterion of cell death. Thus he found that cells in tissue cultures of the rabbit's spleen which had been treated with thorium X stained diffusely with trypan blue. He found no acid dye which would stain the cell nuclei in control cultures, but after treating them with benzol, vital nuclear staining resulted. Wallbach thus came to the conclusion that diffuse staining with acid dyes is characteristic of certain states of functional activity of cells.

In discussing the work on the segregation of basic dyes, reference was made to the work of Nassonov (1930, 1932) who showed that in epithelial cells of the frog's intestine either neutral red was segregated in droplets or a diffuse coloration of the cytoplasm with nuclear staining occurred, according to the state of metabolic activity of the cells. Similar results have recently been published by Alexandrov (1932) who has worked with the larva of *Chironomus plumosus* and *Daphnia pulex*. He concluded that vital staining with basic dyes does not depend upon the properties of the dyestuffs employed (either granular or diffuse-staining colour stuffs), but is conditioned by the state of vital activity of the cells.

IX. SUMMARY.

Certain acid dyes (*e.g.* trypan blue) are segregated by many living cells in the form of droplets or granules. Segregation *in vivo* has been shown to be influenced by the rate of diffusion of dyestuffs: but experiments carried out *in vitro* do not afford evidence of any relationship between segregation and rate of diffusion of dyes. Recent researches indicate that the state of functional activity of cells, and also the chemical constitution of the dyestuffs may be of fundamental importance.

Neither *in vivo*, nor *in vitro* do malignant cells of the mouse segregate acid dyes in the same manner as their non-malignant prototypes: but malignant cells phagocytose particulate matter which may have been coloured by dyes.

Only rarely are preformed cell inclusions stainable with acid dyes, but most cytoplasmic vacuoles (not fat droplets) and many kinds of granules are stainable with basic dyes, such as neutral red and brilliant cresyl blue. Such staining is evidently the result of the flocculation of the dye by the more acid cytoplasmic inclusions.

This type of staining has been shown to be influenced by the state of functional activity of the cells.

Basic dyes can also be segregated like acid dyes, but the process does not occur under anaerobic conditions. Janus green is the only dye which has been shown to stain mitochondria universally. No specific stain has been found for the Golgi bodies. The living nucleus apparently only stains under special metabolic conditions.

In some cells of a secretory, and of an excretory function the segregation of dye seems to be brought about by the Golgi apparatus.

Basic dyes, which are readily soluble in lipins, and not flocculated in contact with acid colloids (*e.g.* rhodamine B), stain cells diffusely. Other basic dyes (*e.g.* neutral red), have also been found to stain living cells diffusely in certain states of functional activity (*e.g.* anaerobiosis). Acid dyes have been found to stain cells diffusely under abnormal conditions (*e.g.* action of thorium X and benzol), besides staining dying and dead cells.

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STIMULATIONSORGANE[1]

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(Mit zehn Abbildungen.)

INHALT.

I. EINLEITUNG.

Das Problem der Stimulationsorgane ist eine der schwierigsten Fragen der heutigen vergleichenden Physiologie. Der Begriff befindet sich noch im Embryonalstadium, und ist noch in den meisten Punkten sehr labil begründet, oder strittig. Die Tatsachen, die zur Bildung desselben führten, sind grösstenteils solche, die

nicht in die Rahmen der klassischen Physiologie passen, und werden deshalb meistens einfach übersehen, oder bleiben unbeachtet. Dies erklärt auch, weshalb die diesbezüglichen Kenntnisse so lückenhaft und unsicher sind.

Die folgenden Zeilen sollen sozusagen nur diese Mängel zeigen. Sie können—da sie nur ein Sammelreferat bilden—die Lösung der Probleme gar nicht anstreben. Es ist auch einfach unmöglich, auf Grund unserer heutigen Kenntnisse, ohne weitere, eingehende vergleichende Untersuchungen die Streitfragen des Stimulationsproblemes zu lösen. Wenn es gelungen ist, mit den folgenden Zeilen auf unbeachtete und vernachlässigte Tatsachen hinzuweisen, von welchen man auf das Vorhandensein stimulatorischer Funktionen, bzw. Organe schliessen kann, dann hat das Referat sein Ziel schon erreicht. Es will nichts mehr, als eine Vorarbeit zur experimentellen Lösung der hier geschilderten Probleme sein. Deshalb kommt es, dass im Stoff viele einander widersprechende Auffassungen wiedergegeben sind. Manche werden der Ansicht sein, dass der Verfasser viel mehr Kritik und Auswahl bei der Zusammenstellung des Stoffes hätte ausüben sollen. Eine solche Kritik und Auswahl wurde aber eigentlich in der Tat vorgenommen, nur war es oft unmöglich zwischen einander widersprechenden Angaben den Streit zu entscheiden. Da eine experimentelle Nachprüfung solcher Streitfragen ausserhalb der Aufgaben eines Referates steht, müssten sie einfach offen gelassen werden.

(1) BEGRIFF DER STIMULATIONSORGANE.

Als Stimulationsorgane bezeichnet man Sinnesorgane, denen als Hauptfunktion, oder neben ihrer Hauptfunktion, die Aufgabe zukommt, dem gesamten Nervensystem, oder einzelnen Nervenbahnen dauernd Erregungen (Stimuli) zuzuführen, die ihrerseits aber keine spezifischen Reflexe auslösen, sondern dazu dienen, das gesamte Bewegungssystem ständig in einem gewissen Zustande zu halten, welcher zur normalen Ausführung seiner Tätigkeit unbedingt nötig ist.

Wie aus dieser Definition hervorgeht, haben wir es mit einem, wichtige Funktionen ausübenden System zu tun. Es besteht ja ganz allgemein ein inniger Zusammenhang zwischen dem Sensorium und dem Motorium des Organismus; die Funktionen, die das Muskelsystem ausführt, und dementsprechend der jeweilige Zustand desselben hängt ja mehr oder minder von den äusseren Reizen ab, die wieder durch die Sinnesorgane rezipiert werden.

Diese Rezeptionen, d.h. die Erregungen die von den Rezeptionsorganen erzeugt und zentripetal fortgeleitet werden, haben aber verschiedene Wirkungen auf das Bewegungssystem. Während manche Erregungen nur gewisse genau umschriebene plötzliche Reflexe auslösen, werden andere und zwar meistens dauernd bestehende Erregungen mit einem allgemeinen Zustand oder Zustandsänderung des gesamten Muskelsystems beantwortet. Es ist noch fraglich, ob man diese letztere Erscheinung den echten Reflexen scharf gegenüberstellen kann, wie etwa Buddenbrock (1924-28) denkt. Es kann auch angenommen werden (und wird auch von den meisten Physiologen), dass die Wirkungen eigentlich Dauerreflexe sind, also dauernd bestehende geringe Erregungen, die ständig reflektorisch beantwortet werden (vgl.

z.B. Bremer, 1932). Ob dies so ist, oder nicht, kann heute noch nicht entschieden werden. Allerdings gibt es Zeichen dafür dass diese Auffassung nicht ausreicht. Die typischen Stimulationsorgane, z.B. die Halteren, der Fliegen, beeinflussen nämlich nicht nur mit ihnen zusammenhängende, genau abgegrenzte Reflexe, sondern auch solche, mit denen sie nicht in unmittelbarer Verbindung stehen. Eine Fliege, die normalerweise einer Lichtquelle zufliegt, wird nach Halterenentfernung dies nicht tun, obwohl die Lichtsinnesorgane ebenso wie auch die betreffenden Reflexbahnen und Effektororgane (Flügelmuskulatur) vollkommen heil sind. Weiterhin wird bei den Stimulationswirkungen sehr oft Nervenenergie auf längere Zeit gespeichert, während typische Reflexe unmittelbar auf die Erregung folgen, und zwar mit grösster Geschwindigkeit. Trotz dieser Bedenken fehlen aber sehr oft die Unterschiede zwischen typischen Reflexen und Stimulationswirkungen, oder sie sind verwischt, so dass man zwischen beiden zur Zeit keine scharfe Grenze ziehen kann.

Das Wesen stimulatorischer Erscheinungen, und ihre Bedeutung im Leben des Organismus, kann man am besten verstehen, wenn man bedenkt, dass der Ablauf eines Reflexes eigentlich aus zwei Phasen besteht. Zuerst wird durch die zentripetale Nervenbahn die Erregung vom Rezeptionsorgan zum Zentralnervensystem geführt, und dann wird dort durch diese Erregung—wie Buddenbrock (1924–28) sich ausdrückt—"eine Entladung des motorischen Neurons" bewirkt, d.h. eine andere, zentrifugal ablaufende Erregung ausgelöst, welche dem Effektor zufliesst. Matula (1911) hat diesen Prozess mit der Abfeuerung einer Flinte verglichen, wobei die Energie, die zum Anziehen des Hahnes verwendet wird der zentripetalen Erregung des Sinnesorgans, während die Detonation des Pulvers der zentrifugalen Erregung des motorischen Neurons entspricht. Der Ablauf eines Reflexes ist also nicht etwa so zu verstehen, dass die peripher erzeugte Erregung direkt den Effektoren zugeführt wird, vielmehr wird dadurch nur eine andere Energiequelle in Betrieb gesetzt, die im Centrum liegt und centrifugale Erregung erzeugt. Hieraus ergibt sich auch, dass zum normalen Funktionieren der Reflexbögen eine Art "Nervenenergie" nötig ist, auf deren Kosten die nervösen Leistungen ausgeführt werden. Ferner ist "Nervenenergie" auch zur Aufrechterhaltung des normalen Ruhezustandes der Muskeln, und zwar zur Aufrechterhaltung des Tonus nötig[1]. Die "Nervenenergie" für die motorischen Nervenbahnen wird nun von den Stimulationsorganen erzeugt. Diese halten durch die von ihnen ausgehenden, manchmal ganz sinnlos und überflüssig erscheinenden Erregungen den ständigen Tonus des Muskelsystems aufrecht, und liefern die "Nervenenergie," durch welche die zentrifugale Erregung als Folge der zentripetalen zustande kommt.

Im Zentralnervensystem gibt es sog. Erregungszentren, die das gesamte Bewegungssystem, oder gewisse Muskelgruppen mit der zur Aufrechterhaltung seines normalen Zustandes und Funktionierens nötigen Energie versorgen. Diese stellen, nach einer Bezeichnung von Uexküll (1908), sozusagen "Tonusreservoire" dar, da sie von den verschiedenen Stimulationsorganen gespeist werden, und die durch diese gelieferte Energie aufspeichern. Dies betrifft natürlich aber nicht die

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[1] Es soll hier allerdings die Frage offen gelassen werden, ob es auch andere Quellen, und Ursachen des Vorhandenseins eines Muskeltonus existieren (vgl. hierzu v. Uexküll, 1926).

sog. automatischen Erregungszentren, von deren Energiequellen wir zur Zeit noch so gut wie nichts wissen.

Auf Grund des gesagten kann man die Kriterien stimulatorischer Funktionen folgendermassen formulieren:

1. Lieferung solcher Erregungen, die keine echten Reflexe auslösen, sondern zur Aufrechterhaltung des normalen Zustandes (d.h. des normalen Tonus, der normalen Bewegungskraft, und -geschwindigkeit, u.s.w.) des Bewegungssystems dienen.

2. Lieferung solcher Erregungen, die auf die Dauer bestehen.

3. Ausbreitung der Wirkung auf das ganze Bewegungssystem, oder wenigstens auf grössere Körperregionen.

4. Vorhandensein spezieller Zentren zur Aufspeicherung der stimulatorischen Erregungen.

Allerdings muss hierzu bemerkt werden, dass von diesen Kriterien nur das erste einen absoluten Beweis für das Vorhandensein einer Stimulationswirkung bildet. Es ist aber oft ganz unmöglich festzustellen, ob eine Erscheinung Tonuserzeugung, bzw. andere ähnliche Zustandsbeeinflussung, oder aber ein gewöhnlicher reflektorischer Prozess ist. In solchen Fällen muss man die weiteren, indirekten Beweise (Kriterien 2, 3 u. 4) heranziehen. Diese sind aber keine absolute Kriterien der stimulatorischen Erscheinungen, und so spricht auch ihre Abwesenheit noch nicht gegen das Vorhandensein von Stimulationswirkungen. (Zum Beispiel finden wir in manchen Fällen stimulatorische Erscheinungen ohne Erregungszentren.)

Man kennt "Sinnesorgane," die nach der heutigen Auffassung ausschliesslich solche Erregungen produzieren, die zur Erhaltung des normalen Zustandes der Bewegungsorgane dienen, also spezifische Stimulationsorgane darstellen. Es kommt aber auch solchen Sinnesorganen, die eine ganz andere Hauptfunktion von höchster Wichtigkeit besitzen, z.B. den Augen, nebenbei noch eine Stimulationsfunktion zu, und man kann auch diese mit Recht als Stimulationsorgane betrachten. Sogar jene Sinnesorgane, über deren Stimulationswirkung heute noch gar nichts bekannt ist, kann man nicht aus dem Begriff endgültig ausschalten, da die Möglichkeit immer besteht, dass ähnliche Funktionen auch bei diesen nachgewiesen werden.

(2) Entwicklung des Begriffes.

Die geschilderte Formulierung des Begriffes der Stimulationsorgane ist noch verhältnismässig neu, obwohl die Tatsachen, aus denen der Begriff konstruiert wurde, schon seit längerer Zeit bekannt sind. Abgesehen von den längst bekannten Erscheinungen der Tonusfunktion des Wirbeltierlabyrinthes, waren hauptsächlich einige Erscheinungen der Nerven- und Muskelphysiologie niederer Wirbelloser, die zur Bildung des Stimulationsbegriffes führten. Besonders die Frage nach den Reizquellen des ständigen Muskeltonus, und gewisser scheinbar ganz automatischer Bewegungen, wie die rhythmischen Kontraktionen der Medusen, die Atembewegungen der Libellen, u.s.w. führten zur Vorstellung von solchen "Erregungsorganen," oder "tonischen Sinnesorganen" die für die Lieferung der nervösen

Energie zu diesen Bewegungserscheinungen verantwortlich gemacht werden
können. I. v. Uexküll war einer der Bahnbrecher auf diesem Gebiet, und ein Vor-
kämpfer der geschilderten Ansichten. In seinen Untersuchungen über die Rand-
körper der Meduse *Rhizostoma pulmo* (1901), auf die wir noch zurückkommen
werden, hat er den Begriff des Stimulationsorgans schon mit grosser Klarheit
abgefasst, und auch in seinen verschiedenen späteren Arbeiten, auf die wir uns an
gegebener Stelle noch berufen werden, hat er zu diesem Begriff weitere Beiträge
geliefert. Ähnliche Gedanken hat Jordan in seinen Arbeiten über die Physiologie
des Nervensystems der Pulmonaten (1906) und über reflexarme Tiere (1908, 1912)
ausgeführt (vgl. ferner Jordan u. Hardenberg, 1926). Auch Untersuchungen von
Fröhlich u. Löwi (1907) an *Eledone*, ferner von Matula (1911) an Libellen, haben die
Ausbildung des Stimulationsbegriffes weiter gefördert. Obwohl die letzteren später
bezweifelt wurden (z.B. Wallengren, 1913-14; Stahn, 1928), waren sie sehr anregend
für weitere Untersuchungen. Buddenbrock gebührt der Verdienst, den Begriff der
Stimulationsfunktion, und seiner organischen Grundlagen zu einem vollständigen
System ausgebaut zu haben. Abgesehen von seinen früheren Studien über die toni-
sierende Wirkung der Statocysten (1912, 1913) und Augen (1919a), hat er sich
hauptsächlich bei seinen Untersuchungen über die Bedeutung und Funktion der
Halteren der Dipteren (1917, 1919b) mit dem Stimulationsproblem theoretisch und
experimentell eingehend beschäftigt. Den Inhalt des Begriffes erweiternd hat er sich
den Ansichten der obengenannten Forscher angeschlossen. Sein Schüler Lehmann
(1922) hat sich auf ähnliche Weise mit den Sinnesorganen verschiedener Medusen
beschäftigt. Schon früher, und inzwischen mit wiederholter Betonung, wurde
gezeigt (z.B. Rádl, 1903; Garrey, 1918, u.a.) dass gewisse Funktionen der Augen
denen der sog. tonischen Sinnesorganen vollkommen entsprechen, dass also auch
diese, im Leben des Organismus eine so wichtige Rolle spielenden Sinnesorgane
nebenbei noch als "tonische Sinnesorgane" funktionieren. Dadurch war die
Möglichkeit gegeben, den Begriff zu erweitern, ja sogar verallgemeinern. Den
Namen "Stimulationsorgan" hat Buddenbrock zuerst in seinem hervorragenden
Grundriss der vergleichenden Physiologie (1927-28) benutzt, und die daselbst aus-
geführten Erwägungen (S. 99-102, 122-123, 183-188, etc.) haben dem Begriff den
heutigen Inhalt und die heutige Form gegeben. Bozler (1926a) hat sich in dem
theoretischen Teil seiner Arbeit über das Ocellenproblem mit der Stimulations-
funktion in demselben Sinne beschäftigt.

Betreffs der Art und Weise, wie die Stimulationsorgane ihre Wirkung auf das
Bewegungssystem ausüben, hat Bozler sich aber nicht der früheren Auffassung
angeschlossen. Die Beeinflussung des allgemeinen Bewegungssystems besteht
nämlich, wie erwähnt, einerseits aus einer ständigen Regulation des Muskeltonus,
andererseits aber aus einer Steigerung und Beschleunigung der Bewegungen, d.h.
der Muskelkontraktionen. Früher wurden alle diese Wirkungen als Tonus
bezeichnet, dies ist aber—wie sich Bozler ausdrückt—"per definitionem falsch,"
und beruht auf einem Missbrauch des Tonusbegriffes. Es ist hier nicht der Platz
die bekannten, aber immer noch so rätselhaften Tatsachen über die zweifachen
Funktionen der Skelettmuskulatur zu erörtern, es ist aber allbekannt, dass es ein

scharfer Unterschied zwischen den zwei Funktionsweisen, den plötzlichen Bewegungskontraktionen und tonischen Dauerkontraktionen, zwischen Bewegung und Sperrung besteht, die man bis heute noch nicht auf einen gemeinsamen Nenner bringen konnte. Als Tonus sollten richtigerweise nur jene Erscheinungen bezeichnet werden, welche sich auf die zweiterwähnte Funktion der Muskeln beziehen. Es ist aber schon von vornherein unwahrscheinlich, dass die verschiedenen Stimulationsorgane nur diese eine Funktion des Muskelsystems beeinflussen sollten. Die die Muskelbewegungen betreffende Stimulationsfunktion kann dann aber nicht als Tonuserzeugung bezeichnet werden, wie es früher geschehen war, sondern muss davon irgendwie unterschieden werden. Bozler hat für diese letztere Funktion die Benennung "kinetische Stimulation" vorgeschlagen, und die Wirkung selbst als "Kinese" bezeichnet. (In seinem speziellen Falle "Photokinese," ein Fachausdruck, welchen Engelmann (1883) für gewisse Bakterien schon früher benutzt hat.) Er will dementsprechend zwischen tonuserzeugenden, bzw. kinetischen Stimulationsorganen unterscheiden.

Diese Auseinanderhaltung beider Funktionen ist durchaus berechtigt, sie kann aber meistens nur sehr schwer durchgeführt werden. Nehmen wir z.B. wie es oft beschrieben wird, eine allgemeine Muskelschwäche als Ausfallerscheinung nach Exstirpation eines Stimulationsorgans an. Handelt es sich dann um eine tonische Schwäche, d.h. ein Tonusverlust des Muskelsystems, oder um eine kinetische Schwäche, d.h. Verlust der Fähigkeit Muskelkontraktionen in normaler Anzahl und mit normaler Kraft auszuführen, oder aber um beide Erscheinungen gleichzeitig? Man muss annehmen, dass die meisten Stimulationsorgane sowohl eine tonische, wie auch eine kinetische Wirkung ausüben, und wenn auch bei vielen eine dieser Funktionen überwiegt, gibt es wieder andere, bei denen beide die gleiche Bedeutung und Wichtigkeit haben.

Dementsprechend werden wir die von Bozler vorgeschlagene Aufteilung der Stimulationsorgane auf tonuserzeugende, bzw. kinetische nicht beibehalten, und werden nur innerhalb der einzelnen Abschnitte, wo es überhaupt möglich ist, die tonischen, bzw. kinetischen Stimulationsfunktionen gesondert behandeln.

Wir wenden uns zuerst zu den sog. niederen Sinnesorganen, um zu sehen, was für stimulatorische Wirkungen diese auf das Nervenmuskelsystem ausüben. Dann werden wir die hauptsächlich tonisierende Wirkung der statischen Sinnesorgane besprechen, und endlich die verschiedenen Stimulationsfunktionen, die die Lichtsinnesorgane ausüben, erörtern.

II. BESCHREIBUNG DER STIMULATIONSERSCHEINUNGEN.

(1) DIE STIMULATIONSFUNKTION NIEDERER SINNESORGANE.

Als "niedere Sinnesorgane" sollen nach Baglioni (1913 b) diejenigen bezeichnet werden, die von verhältnissmässig einfachem Bau sind und keine komplexen Apparate mit allerhand Hilfsapparaten darstellen, wie z.B. die statischen Sinnesorgane, oder die Augen. Sie sind also nicht aus einer grösseren Anzahl systematisch geordneter Sinneszellen aufgebaut, es fehlen ihnen die verschiedenen Hilfsapparate, mit denen

zusammen sie zu höheren Einheiten verbunden sind. Sie sind also im allgemeinen durch Einfachheit und losen Zusammenhang der einzelnen Teile charakterisiert. Man rechnet hierher die verschiedenen mechanischen Sinnesorgane (Tast-, Druck-, Erschütterungssinn, u.s.w.), die Temperatursinnesorgane, die Organe des Schmerzsinnes, ferner die Geruchs- und Geschmacksorgane, d.h. die sog. chemischen Sinnesorgane, und endlich auch die verschiedenen stiftführenden Sinnesorgane der Insekten (Chordotonalorgane, Johnstonsche Organe, Tympanalorgane, u.s.w.), die zwar schon ziemlich hochorganisierte Gebilde sind, jedoch nach Natur, Ursprung, und wahrscheinlicher Funktion unter diesem Begriff einzureihen sind.

Verschiedenen Gruppen der niederen Sinnesorgane kommt nun neben ihren verschiedenen Hauptfunktionen auch eine mehr oder minder ausgesprochene Stimulationsfunktion zu. Das meiste was man hierüber weiss, betrifft die mechanischen Sinnesorgane im allgemeinen; dann kommen noch chemische und thermische Sinnesorgane einigermassen in Betracht, und endlich sind noch einige, zu dieser Gruppe gehörende Sinnesorgane, die als spezifische, im Dienste der Dauerbeeinflussung des Bewegungssystems stehende Stimulationsorgane betrachtet werden. Wir wenden uns danach zuerst zur Gruppe der mechanischen Sinnesorgane, dann werden die Stimulationsfunktionen anderer niederer Sinnesorgane besprochen, und endlich soll die Frage erörtert werden, ob auch spezifische Stimulationsorgane unter den sog. niederen Sinnesorganen vorkommen.

A. *Mechanische Sinnesorgane.*

(a) Stimulationsfunktion in engerem Sinne.

Die tonuserzeugende Stimulationsfunktion mechanischer Sinnesorgane wurde hauptsächlich bei höheren Wirbeltieren und beim Menschen nachgewiesen und eingehender untersucht. Der eigentliche Sitz dieser Tonuserzeugung ist dabei aber nur ungenau lokalisiert, und einige Erscheinungen lassen darauf schliessen, dass eigentlich alle mechanischen Sinnesorgane eine solche Funktion ausüben. Hauptsächlich kommen jedoch diejenigen Sinnesorgane in Betracht, die zur Empfindung der Lage und der Bewegungen einzelner Körperteile, also dem sog. Muskelsinne dienen ("propriozeptiver Sinn," vgl. Sherrington, 1906).

Die tonuserzeugende Funktion der mechanischen Sinnesorgane äussert sich am klarsten in den Erscheinungen des sog. Reflextonus. Damit bezeichnet man die Erscheinung, dass dem Zentralnervensystem (Rückenmark) zuströmende periphere Erregungen (erzeugt durch verschiedene sensible Endigungen) eine tonisierende Wirkung auf die Muskulatur ausüben. Ein Beispiel hierfür liefert das Brondgeestsche Phänomen, welches darin besteht, dass nach Durchschneidung der sensiblen Nerven der Extremitäten der Tonus derselben fast völlig verschwindet, obwohl die motorischen Bahnen unversehrt bleiben. Man nimmt am besten dekapitierte Frösche zum Zeigen dieser interessanten Stimulation. Wenn man diese nach einer Zeit am Kopfende aufhängt, werden die Hinterbeine nicht schlaff herabhängen, sondern eine Stellung einnehmen, die mehr der normalen Hockstellung ähnelt, also es besteht ein gewisser Tonus in den Extremitäten. Der Stimulus desselben ist nun

die Summe jener zentripetalen Erregungen, die in der Haut und in den Muskeln infolge der abnormalen Lage der Extremitäten zustande kommen. Dass dies tatsächlich so ist, beweist dann die Durchtrennung der sensiblen Nerven, d.h. der Hinterwurzel der Spinalnerven, also eine Aufhebung afferenter Erregungen. Durch dieses Durchschneiden wird ein schlaffes Herabhängen der Extremität verursacht, trotz des ungestörten Funktionierens der efferenten motorischen Bahnen. Durch eingehende Untersuchungen hat Trendelenburg (1906) ähnliches an Tauben gezeigt (Abb. 1). Benjamins u. Huizinga (1928) haben den Tonusverlust auch graphisch registriert. Nach Spiegel u. Worms (1927) üben bei der Katze die Sinnesorgane der Eingeweide einen ähnlichen stimulatorischen Einfluss auf dem Tonus der Extremitäten aus.

Ähnliches, wie mit Hinterwurzeldurchschneidung, kann erreicht werden, wenn nicht die afferenten Nervenbahnen, sondern die stimulierenden Sinnesorgane selbst ausser Funktion gesetzt werden. Dies kann z.B. durch Narkotisierung mit Novocaininfiltration erreicht werden, wobei der Tonus der Muskulatur stark herabgesetzt wird (Liljestrand u. Magnus, 1919, Bremer u. Titeca, 1930).

Klinische Fälle zeigen auch Beweise für das Vorhandensein des Reflextonus, d.h. der Stimulationswirkung mechanischer Sinnesorgane. Bei Krankheiten, bei welchen afferente Nerven gelähmt sind, entsteht sehr oft eine recht auffallende Tonusverminderung. So z.B. bei *Tabes dorsalis*, wobei die zentripetalen Bahnen stark angegriffen werden. Es ist eine schöne Anerkennung der Stimula-

Abb. 1. Beinhaltung einer an den Flügeln frei gehaltenen Taube. Die Hinterwurzeln des rechten Beines sind durchgeschnitten, das Bein hängt wie leblos herab. Nach Trendelenburg.

tionswirkung mechanischer Sinnesorgane, dass in solchen Fällen sehr oft verschiedene Bandagen, Gurte, u.s.w. für die heilen Körperteile verschrieben werden, um durch diese die Stimulationswirkung zu verstärken, und den Verlust an Tonus einigermassen zu kompensieren. Bei anderen Krankheiten entsteht dagegen eine Tonuserhöhung, wie bei übergrosser Reflexerregbarkeit des Rückenmarkes in der Littleschen Krankheit. Diese wurden manchmal mit Erfolg so behandelt, indem man einige sensible Nerven durchschnitt (vgl. Höber, 1931). Über weitere Literatur solcher Erscheinungen siehe Bremer (1932).

Auch bei Wirbellosen kennt man eine tonuserzeugende Funktion mechanischer Sinnesorgane. Die Organe, die für diese Funktionen verantwortlich gemacht werden können, sind verständlicherweise nicht näher lokalisiert. Die verschiedensten Sinneshaare, die in der Haut der Arthropoden ganz allgemein vorkommen, ferner die Sinnesknospen der Haut der Würmer, u.s.w. können dafür alle in Betracht kommen. Ob speziell propriozeptiven Sinnesorganen bei Wirbellosen eine ähnliche spezifische Stimulationsfunktion zukommt, wie bei höheren Wirbeltieren, ist zur Zeit noch nicht bekannt. Man weiss überhaupt noch sehr wenig über solche Sinnesorgane, obwohl die Fähigkeit verschiedener Tintenfische, Krebse, u.s.w. die

Bewegungen ihrer Körperteile (Arm, Schere) in bestimmter Richtung lenken zu können, allgemein bekannt ist, was auf das Vorhandensein propriozeptiver Reize schliessen lässt. Für diese Fähigkeit werden im allgemeinen die verschiedenen Hautsinnesorgane verantwortlich gemacht. Bei Arthropoden können hierfür die in der Umgebung der Gelenke sich befindenden Sinneshaare in Betracht kommen. Es ist nun interessant, dass verschiedene stiftführende Sinnesorgane, denen bereits Buddenbrock eine mutmassliche Stimulationsfunktion zugeschrieben hat (1919 b, 1924–28), manchmal auch als propriozeptive Sinnesorgane gedeutet werden.

Von Beobachtungen, die für das Vorhandensein einer tonischen Stimulations-funktion mechanischer Sinnesorgane bei Wirbellosen sprechen, seien nur die von Uexküll (1895) bei dem Rohrwurm *Sipunculus* erwähnt. Es wurde nachge-wiesen, dass dieses Tier, wenn es längere Zeit in seiner Sandröhre zurückgezogen liegt, seinen Tonus fast vollkommen verliert, und dieser erst nach mehreren mechanischen Reizungen wieder auftritt. Diese Erscheinung weist darauf hin, dass die durch die mechanischen Reize verursachten Erregungen zur Erhaltung des normalen Muskeltonus nötigen Stimuli darstellen, und bei anhaltendem Ausbleiben dieser Erregungen der Tonus allmählich verloren geht. Der Schwirrflug Nacht-schmetterlinge nach dem Tagschlaf, und andere in dieser Kategorie gehörende Erscheinungen werden von Buddenbrock (1917, 1924–28) in ähnlichem Sinne gedeutet, obwohl hierfür noch keine experimentellen Beweise vorliegen.

Eine kinetische Stimulationsfunktion mechanischer Sinnesorgane wird von Matula (1911) auf Grund seiner Versuche an Libellenlarven postuliert. Diese Tiere sollen die "Nervenenergie," die zur Ausführung der Atembewegungen, d.h. der rhythmischen Bewegungen des Abdomens nötig ist, aus Erregungen schöpfen, die von den mechanischen Sinnesorganen der Tarsalglieder der ersten Paar Beine produziert werden. Diese Erregungen sollen vom ersten Thorakalganglion, also von einem Tonusreservoir gespeichert, und dem eigentlichen Zentrum des At-mungsreflexes, d.h. dem Ganglion des letzten Körpersegmentes nach Bedarf zugeführt werden. Dies wird deshalb angenommen, weil eine Durchtrennung des Nervenstranges zwischen Thorakal- und Analganglion ein plötzliches Einstellen der Atembewegungen zur Folge hat, während Abschneiden der Tarsen der Beine, also eine Entfernung der eigentlichen Stimulationsorgane unter Beibehaltung des "Tonusreservoirs," eine langsame, allmähliche Abnahme der Atembewegungen verursacht, welche aber endlich gleichfalls zum vollständigen Stillstand führt.

Diese Versuche wurden später von Wallengren (1913–14), und neuerdings von Dotterweich (1928), und Stahn (1928) wiederholt, aber die Ergebnisse leider nicht bestätigt werden können. Heute gelten also die Ergebnisse von Matula als wider-gelegt (Fränkel, 1932). Doch ist die Frage nach der Energiequellen der Atembe-wegungen noch völlig ungelöst. Es wird angenommen, dass die Reize für diese zentral und automatisch entstehen, es ist aber nachgewiesen, dass die Atembewe-gungen nicht nur von chemischen Reizen (CO_2, resp. O_2-Einwirkung: Babák, 1912; Buddenbrock u. Rohr, 1922), sondern auch von mechanischen Reizen, z.B. vom Fluge (Fränkel, 1932 an *Vespa*) beeinflusst, und zwar gesteigert werden. Dies ist allerdings von grösster Wichtigkeit, und vor weiteren Untersuchungen darf die

Matulasche Auffassung über die Stimulationswirkung der mechanischen Sinnes-
organe der Insektenbeine nicht vollkommen aufgegeben werden. Umso weniger,
als die Versuche von Buddenbrock (1919b) zur Analyse der Halterenfrage bei
Sarcophaga nebenbei auch die Stimulationsfunktion der mechanischen Sinnes-
organe der Beine wieder bewiesen haben (s. weiter unten).

(b) Erscheinungen der Stereokinese.

Neben diesen Erscheinungen kommt den mechanischen Sinnesorganen eine
eigenartige kinetische Funktion zu, die in der Auslösung typischer ungerichteter
Bewegungsreaktionen besteht und neuerdings Stereokinese (Fraenkel, 1931) ge-
nannt wird. Diese Erscheinung wurde früher allgemein als Thigmotaxis oder
Stereotaxis bezeichnet, hat aber mit der Orientierung unmittelbar nichts zu tun,
und stellt nur einen allgemeinen Erregungszustand des Bewegungssystems dar, der
von mechanischen Reizen hervorgerufen wird. Es muss hier allerdings bemerkt wer-
den, dass es nicht sichergestellt ist, ob die stereokinetischen Erscheinungen stimu-
latorischer Natur sind. Man kann ebenso gut annehmen, dass viele, vielleicht sogar
alle nur einfache Reflexe darstellen. Da aber die Wirkung sich meistens auf das ganze
Muskelsystem ausbreitet (die Reaktionen erscheinen ganz ungerichtet), kann man
annehmen, dass nicht gewisse genau umschriebene Reflexbahnen, sondern echte
Erregungszentren durch die Erregungen in Betrieb gesetzt werden, also Stimula-
tionswirkungen im Spiel sind. Allerdings harrt diese Annahme der experimentellen
Nachprüfung.

Die Erregungen, die diese eigenartige kinetische Wirkung ausüben, entstehen
eigentlich durch ein Ausbleiben mechanischer Reize, wie es an verschiedensten
Tieren festgestellt wurde. Die Tiere befinden sich in der Ruhelage so lange, bis
gewisse mechanische Sinnesorgane, meistens die der Extremitäten mit festen
Gegenständen in Berührung stehen. Sobald aber diese Berührung aufgehoben
wird, und die Sinnesorgane ihre gewöhnlichen Reize verlieren, beginnt eine allge-
meine ungerichtete Bewegung, welche so lange dauert, bis die mechanischen
Sinnesorgane wieder Kontaktreize bekommen. Verworn (1889) nennt diese Erschei-
nung "thigmotaktische Fesselung" des Tieres.

Das Verkriechen gewisser Tiere ins Gebüsch, unter Steine, u.s.w. ("Flucht-
reaktionen") ist wahrscheinlich auf solche Reaktionen zurückzuführen. I. v. Uexküll
(1908) hat die stereokinetische Wirkung mechanischer Sinnesorgane bei Libellen
sehr elegant nachgewiesen. Eine am Thorax befestigte Libelle führt, wenn sie frei
schwebend gehalten wird, mit den Flügeln heftige Schwirrbewegungen aus.
Sobald aber zwischen die Füsse ein Papierkügelchen, oder dergleichen geschoben
wird, den dort befindlichen mechanischen (taktilen) Sinnesorganen also ein Kon-
taktreiz geboten wird, stellen die Flügel ihre Bewegungen ein. Auch bei anderen
Insekten, ferner Krebsen (z.B. Alverdes, 1926) und Würmern (vgl. Herter, 1929), ist
eine ähnliche "thigmotaktische Fesselung" bekannt, die oft mit anderen Reak-
tionen, z.B. mit Phototaxis interferiert (Alverdes, 1926; Wolsky u. Huxley, 1932).

Auch gewisse Umkehrbewegungen, d.h. geotaktische, vom Lagesinn (statischen
Sinnesorganen) bedingte Reflexe werden durch stereokinetische Reize stimuliert,

z.B. bei Insekten. Wie bekannt, führen diese die Umkehrsbewegungen nur dann
aus, wenn ihnen die mechanischen Reize für die taktilen Sinnesorgane der Beine
fehlen. Wenn man aber die Beine mit ein festes Gegenstand in Berührung bringt,
so können die Tiere in abnormaler Lage festgehalten werden.

Ein ähnlicher Mechanismus befindet sich nach Buddenbrock (1912) bei ge-
wissen Echinodermen. Die Holothurie *Synapta digitata*, welche im Meeressande
vergraben lebt, führt heftige geotaktische Bewegungen aus, sobald der Kontakt der
Leibesoberfläche mit dem Sand aufgehoben wird. Wenn das Tier sich aber wieder
vergraben hat, werden die geotaktischen Bewegungen automatisch eingestellt.

Bei Nacktschnecken (*Limax, Agrion*), ferner bei Gehäuseschnecken (*Helix*), hat
Baunacke (1913) eine derartige Wirkung der Fussohle nachgewiesen. Die Befreiung
der Sohle vom Untergrund ruft sofort heftige Bewegungen der Muskulatur hervor,
die aber gerichteter, und zwar geotaktischer Natur sind, und von der Statocyste
reguliert werden. Sobald aber die Fussohle wieder Kontaktreize erhält, werden
diese geotaktischen Bewegungen völlig eingestellt, auch wenn das Tier noch weiter
in abnormaler Lage ist. Es ist eine ganz typische stimulatorische Erscheinung,
der wir auch noch später, z.B. bei der photokinetischen Stimulation begegnen
werden, dass die von den Stimulationsorganen erzeugten Erregungen nicht dem
ganzen Muskelsystem zufliessen, sondern ihre Wirkung vermittels irgend eines
anderen Sinnesorgans—dessen Funktion durch die Stimuli verstärkt wird—ausüben.

Die Untersuchungen von Crozier u. Moore (1923) und Crozier (1924) an
Diplopoden und Mehlmottenlarven haben gezeigt, dass die stereokinetische Wir-
kung der einzelnen mechanischen Sinnesorgane sich nicht auf den ganzen Körper
bezieht, sondern dass die an verschiedenen Körperseiten liegenden Sinnesorgane nur
je eine Körperseite beeinflussen, gerade so, wie die meisten Gleichgewichtsorgane,
deren Stimulationsfunktion ebenfalls nur auf je eine Körperseite ausgeübt wird
(s. weiter unten). Dadurch können sogar gewisse Kreisbewegungen erzielt werden,
wenn nämlich Kontaktreize nur an der einen Seite vorhanden sind und diese Reize
dann plötzlich aufgehoben werden. Dann erfolgt eine Wendung nach dieser Seite,
welche offenbar durch eine Erhöhung der kinetischen Wirkung der Hautsinnes-
organe auf der entgegengesetzten Seite verursacht wird. Solche Überkreuzung der
Leitungsbahnen werden wir merkwürdigerweise auch bei der Stimulationswirkung
statischer und photischer Sinnesorgane antreffen.

Crozier u. Pincus (1927) haben ähnliche Erscheinungen auch bei höheren
Wirbeltieren, und zwar bei Ratten und Mäusen nachgewiesen. Junge Tiere, deren
Augen noch nicht geöffnet sind, ferner solche unmittelbar nach der Augenöffnung,
sowie eine experimentell erzeugte blinde Rasse (Mäuse mit sehzellenloser Retina)
zeigen in ihrem Benehmen eine auffallende Übereinstimmung mit den diesbezüg-
lich untersuchten Arthropoden.

Es handelt sich aber bei den von Crozier und Mitarbeitern beschriebenen
Erscheinungen nicht ausschliesslich um stereokinetische Erscheinungen, sondern
auch noch um weitere Faktoren (z.B. sog. homostrophische Reflexe, d.h. solche mit
denen sich die Tiere, wenn ihr Hinterende eine passive Lageänderung erfährt, in
der Richtung derselben einstellen). Allerdings können diese Beobachtungen nicht

so einfach und nicht ausschliesslich auf eine primäre, vom Ausbleiben mechanischer Reize verursachte Stereokinese zurückgeführt werden, wie dies sich Fränkel (1931) vorstellte. Eben hier ist auch das ziemlich zweifelhaft, ob die stereokinetischen Reaktionen tatsächlich Stimulationswirkungen darstellen. Vielmehr kann man typische Reflexe vermuten.

B. *Die Stimulationsfunktion anderer niederer Sinnesorgane.*

Von der Stimulationsfunktion nichtmechanischer niederer Sinnesorgane ist zur Zeit nur noch äusserst wenig bekannt. Allerdings haben bei einigen Tieren Beobachtungen gezeigt, dass Reize der Umwelt unter Umständen nicht mit genau abgegrenzten Reflexen, sondern mit sog. ungerichteten Bewegungsreaktionen, mit einer allgemeinen Erhöhung der Beweglichkeit beantwortet werden. Diese können als Chemo-, Hydro-, Thermokinese, etc. bezeichnet werden. Da diese meistens das ganze Muskelsystem betreffen, ist es möglich, dass sie auf stimulatorischem Wege, mittels gewisser Erregungszentren zustande kommen. Ähnlich können auch tonische Wirkungen hervorgerufen werden, deren Vorhandensein ja durch die Tropismenlehre auch postuliert wird, allerdings aus anderen Gründen.

Obwohl spezielle Untersuchungen über diese noch so sehr unklaren Probleme bis jetzt nicht durchgeführt wurden, kann man aus einigen Untersuchungen auf das Vorhandensein solcher stimulatorischen Funktionen schliessen. So wird von Matthes (1924) über das Geruchsvermögen von Triton geschrieben, dass als Folge des Vorhandenseins chemischer Reize die Tiere spezifische "Witterungsstellungen" einnehmen. Der Körper wird gekrümmt emporgehoben, die Beine gestreckt, so dass sie nur mit den Spitzen den Boden berühren ("Ballettstellung"). Obwohl die Untersuchungen über die letzten Ursachen dieser Zustände nichts aussagen, scheint es sich hier um eine Tonuserhöhung der Gesamtmuskulatur (und bei dem nachfolgenden Herumsuchen vielleicht um eine kinetische Wirkung) zu handeln, die vielmehr stimulatorischer als reflektorischer Natur sein kann.—Auch andere Untersuchungen, die über eine allgemeine Alarmiertheit der Tiere bei Vorhandensein chemischer Reize berichten, weisen darauf hin, dass den chemischen Sinnesorganen neben ihrer Hauptfunktion auch eine stimulatorische Rolle zukommt.

Die verschiedenen Temperatursinnesorgane üben auch ähnliche Funktionen aus. Die eingehenden Untersuchungen von Herter (1924) in den letzten Jahren haben gezeigt, dass verschiedene Tiere in einer sog. Temperaturorgel die optimale Temperatur mit grosser Sicherheit aufsuchen, und dort verweilen. Diese Erscheinung kommt aller Wahrscheinlichkeit nach durch ungerichtete Bewegungen zustande, die in der optimalen Zone einfach eingestellt werden (vgl. Fraenkel, 1931).

Dass solche thermokinetischen Reaktionen tatsächlich vorhanden sind, und als echte stimulatorische Wirkungen betrachtet werden sollen, wurde neuestens von Lang (1932) bewiesen. Dekapitierte Stabheuschrecken (*Carausius morosus*) und Mehlmotten (*Tenebrio molitor*) führen koordinierte Schreitbewegungen aus, die ohne diese Stimulation ausbleiben, wenn man den Untergrund sorgfältig erwärmt (ohne damit die Temperatur der Umgebung bemerkenswert zu erhöhen). Hier sollen durch die Wärme die thermischen Sinnesorgane der Tarsalglieder gereizt werden,

und die Erregungen, die dabei entstehen, den Zentren zugeführt. Es ist dabei bemerkenswert, dass das von Buddenbrock (1921) angenommene Erregungszentrum der Bewegungen, d.h. das Unterschlundganglion, mit dem Kopf auch entfernt ist, und die Stimuli doch wirksam sind, vermutlich deshalb, weil sie unmittelbar den Bewegungszentren, d.h. den Thorakalganglien zufliessen[1].

C. *Die Frage spezifischer Stimulationsorgane unter den niederen Sinnesorganen.*

(a) *Das Problem der Randkörper der Medusen.*

Die Randkörper gewisser Medusen spielten eine wichtige Rolle in der Ausbildung des Stimulationsbegriffes, und der ganze heutige Gedankeninhalt desselben ist
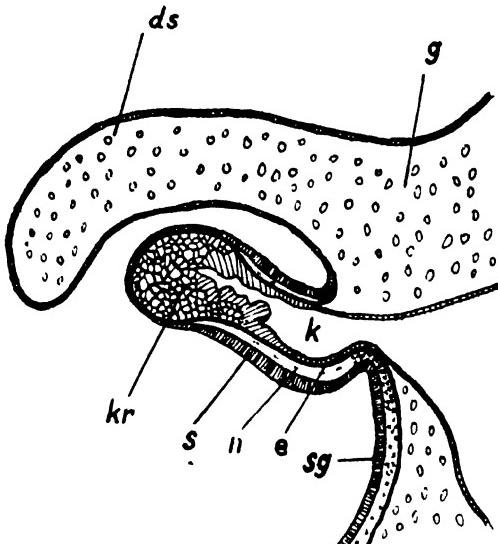
Abb. 2. Längsschnitt durch ein Randorgan von *Cotylorhiza* nach Bozler. *ds*, Deckschuppe; *g*, Gallerte; *kr*, Krystallsack; *s*, Sinnesepithel; *n*, Nervenfilz; *e*, Ektoderm; *sg*, Sinnesgrube.

sozusagen aus Beobachtungen und Versuchen an diesen Sinnesorganen ausgegangen. Bevor wir auf die Besprechung ihrer Funktionen eingehen, müssen einige morphologische Einzelheiten klargemacht werden. Alle diese Ausführungen beziehen sich auf Scyphomedusen, die in der Hinsicht der Randkörperfunktion am meisten untersucht wurden. Der Randkörper bildet eigentlich einen Teil eines komplexen Sinnesorgans, das man als Randorgan bezeichnet, und das mit dem eigentlichen Randkörper sehr oft verwechselt wird (Abb. 2). Die eigentlichen Randkörper sind kolbenförmige Gebilde, die an den Enden der acht radiären und interradiären Gastralkanäle sitzen und sozusagen deren Abschluss bilden. In ihrem Inneren befindet sich eine Fortsetzung der Gastralkanäle, ihr distaler Teil besteht aus dem sog. Krystallsack, d.h. Entodermzellen, die mit krystallinischen Einschlüssen gefüllt sind. Der Krystallsack ist mit dünnem Körperepithel überzogen. Der mittlere und proximale Teil des Randkörpers trägt rund herum ein hohes

[1] Siehe Nachträge, 1, Seite 417.

Zylinderepithel, das von Sinneszellen, nebst Stützzellen aufgebaut ist. Dieses Epithel setzt sich an der subumbrellaren Seite in das Epithel der sog. inneren Sinnesgrube fort, welche aber nicht mehr zum eigentlichen Randkörper gehört. Unter dem Sinnesepithel befindet sich überall ein dichtes Geflecht, welches aus den Fortsätzen der epithelialen Sinneszellen besteht. Dieser Nervenfilz enthält hauptsächlich in der Sinnesgrube zahlreiche Ganglienzellen, die an der Basis des Randkörpers so häufig sind, dass sie den Nervenfilz ganz verdrängen, und mit Recht als ein Ganglion bezeichnet werden. Das Randorgan besteht danach aus dem Randkörper, der inneren Sinnesgrube, und dem Randkörperganglion. Es sei allerdings bemerkt, dass bei vielen Arten die Randkörper an ihren subumbrellaren und exumbrellaren Seite auch verschiedene Ocellen führen, deren Funktion aber von der der eigentlichen Randkörper vollkommen verschieden ist. Auf diese Funktion, und auf die besonders neuerdings viel untersuchte Funktion der Randorgane als Statocysten sei hier nicht eingegangen.

Nun ist schon seit den Untersuchungen von Eimer (1878) und Romanes (1885) bekannt, dass die Randorgane im innigsten Zusammenhang mit den Bewegungserscheinungen der Medusen stehen. Wenn man sämtliche Randorgane wegschneidet, stellt die Meduse alle spontanen Bewegungen plötzlich ein, obzwar sie ihre normale Irritabilität und Bewegungsfähigkeit auch weiterhin behält. Von den Randorganen gehen also offenbar Erregungen aus, die zur Erhaltung der normalen spontanen Bewegungen nötig sind. Eimer hat zwar die Auffassung geäussert, dass diese Erregungen in den Randorganen automatisch gebildet werden, ohne dass dazu von aussen kommende Reize nötig wären, die späteren Untersuchungen haben aber zu der Ansicht geführt, dass die Erregungen dadurch zustande kommen, dass die sehr beweglichen Randkörper durch ihr ständiges Hin- und Herschwingen dauernd Erregungen erzeugen, die auf das Bewegungssystem stimulierend wirken. Diese Auffassung—die erste Formulierung des Begriffes des Stimulationsorgans— wurde hauptsächlich von Uexküll (1901) vertreten, der durch seine schon erwähnten Untersuchungen an der Scyphomeduse *Rhizostoma* dazu gekommen ist. Schon früher hat Romanes (1885) ähnliche Ansichten geäusserst, und die Forscher, die sich nach Uexküll mit der ziemlich viel umstrittenen Frage beschäftigt haben, haben sich fast ausschliesslich dieser Ansicht angeschlossen. Nur einige Meinungen, wie z.B. diese von Baglioni (1913 a), weichen von den geschilderten ab, indem dieser Forscher den Randorganen eine photorezeptorische Funktion zuschreibt. Dies beruht aber, wie Lehmann (1922) nachgewiesen hat, auf einem Irrtum, da Baglioni offenbar die ocellenführenden Randanschwellungen einiger Medusen mit den Randorganen verwechselt hat. Dasselbe gilt für die früheren Untersuchungen von Yerkes (1903) und Yerkes u. Ayer (1903), auf die sich Baglioni bezieht, von denen aber schon Murbach (1903) festgestellt hat, dass sie auf derselben Verwechselung beruhen. Auch die Ergebnisse der Untersuchungen von Jordan (1912) an acraspeden Medusen stehen mit der allgemeinen Auffassung in Widerspruch, worauf wir gleich zurückkommen werden, sie blieben aber leider ziemlich unbeachtet.

Auch der auffallende Rhythmus der spontanen Bewegungen wurde mit der Stimulationsfunktion der Randorgane in Zusammenhang gebracht, indem man

annahm, dass die von diesen erzeugten kontinuierlichen Erregungen durch das periodische Auftreten von Refraktärstadien in eine rhythmische Bewegung verwandelt werden (Romanes, 1885; Bethe, 1910; A. G. Mayer, 1906–8; v. Uexküll, 1901; v. Buddenbrock, 1919 b).

V. Uexküll hat den Einfluss der Randkörper auf die Spontanbewegungen mit einem sehr überzeugenden Versuch nachgewiesen, der zur Grundtatsache aller späteren Erklärungen und Folgerungen wurde. Dieser Versuch bestand darin, dass einer Meduse alle Randorgane bis auf ein einziges weggeschnitten wurden, wobei das Tier aber die Spontanbewegungen noch wie vor, normalerweise ausführte. Wenn nun aber der letzte noch vorhandene Randkörper am Schwingen verhindert wurde, dadurch das man ihn mit irgendeinem Gegenstand festhielt, so blieben die Spontanbewegungen plötzlich aus. Aus dieser Beobachtung wurden dann alle die erwähnten Folgerungen gezogen.

Neuerdings hat sich aber Bozler (1926 b) gegen diese Folgerungen gewendet, und zwar auf Grund seiner Versuche an *Cotylorhiza*. Er hat an dieser Meduse den Uexküllschen Versuch wiederholt, selbstverständlich unter Anwendung aller Feinheiten, die die Verbesserung der Operationstechnik seit zweieinhalb Jahrzehnten entwickelt hat. Als nun nach dem Abschneiden sämtlicher Randorgane in dem letzten übriggelassenen der Randkörper weitgehend zerstört war, hat es sich merkwürdigerweise herausgestellt, dass die Spontanbewegungen unverändert bestehen blieben und sogar ihr Rhythmus sich nicht veränderte. Die Entfernung des Krystallsackes hat gar keine Folgen, das Verletzen des Sinnesepithels verursacht zwar eine plötzliche Schlagfrequenzverminderung, diese verschwindet aber bald wieder vollkommen. Wenn das Sinnesepithel stückweise fein abpräpariert wird, kann man ein Stadium erreichen, in welchem die Frequenzverminderung ständig bestehen bleibt. Die histologische Untersuchung eines so weit zerstörten Randorgans zeigt, dass der Krystallsack, und das ventrale Sinnesepithel völlig entfernt, das dorsale Epithel, und das Randkörperganglion dagegen nur verletzt waren. Nach vollkommenem Ausschneiden des letzten Randorgans kommt eine völlige Lähmung des Tieres zustande.

Bozler zieht aus diesen Versuchsergebnissen die Folgerung, dass der Randkörper in den Randorganen bei dem Zustandekommen der Spontanbewegungen gar keine Rolle spielt. Sein normales Funktionieren ist nämlich schon durch die Entfernung des Krystallsackes völlig zerstört, da dieser den eigentlichen mechanischen Teil des Sinnesorgans bildet. Die Spontanbewegungen müssen nach Bozler vom Randkörperganglion automatisch beeinflusst werden, wie dies schon Eimer (1878) angenommen hat, was aber später bezweifelt wurde. Und hier sollen nun auch die erwähnten Versuchsergebnisse von Jordan (1912) herangezogen werden. Jordan hat nämlich u.a. nachweisen können, dass Stücke von *Rhizostoma octopus* auch nach Entfernung aller Randorgane, ja sogar des ganzen Randes rhythmisch weiterschlagen[1]. Mit dieser, von Bozler leider übersehene Angabe war also nachgewiesen, dass der Medusenkörper schon an sich einen Spontanrhythmus

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[1] Nach einer brieflichen Mitteilung Prof. Jordans gelang es ihm öfters, diese Erscheinung in Kursen zu demonstrieren, aber immer nur an ganz frischem Material.

besitzt wie etwa die Herzkammer der Wirbeltiere. Allerdings gibt Jordan an, dass die Medusen ohne Rand langsamer schlagen als unverletzte Tiere.

Da diese verschiedenen Auffassungen einander in so vielen Punkten widersprechen, muss man die ganze Frage noch mit einer Art Vorbehalt behandeln. Hauptsächlich steht die neueste Auffassung von Bozler mit dem Ergebnis des Grundversuches von Uexküll in Widerspruch. Bozler versuchte zwar dieses damit zu erklären, dass beim Festhalten des letzten Randkörpers im Uexküllschen Versuch das Randkörperganglion eine Art Schock erlitt, wodurch die spontanen Bewegungen plötzlich eingestellt wurden. Diese Annahme ist aber, wie auch Bozler zugibt, nur sehr wenig begründet. Man muss allerdings weitere Versuche, bzw. Nachprüfungen abwarten, bis man sich ein endgültiges Urteil über das Randkörperproblem bilden kann.

(b) Die Halterenfrage.

Die anderen, hier zu besprechenden Sinnesorgane sind die Halteren der Zweiflügler. Die Halterenfrage ist ebenso wie das Ocellenproblem eine der Streitfragen der Insektenbiologie, da es sich—hier wie dort—um eigenartige Organe

Abb. 3. Längsschnitt durch eine Haltere. Nach Pflugstädt.

handelt, die keine Ähnlichkeit, und keinen Vergleichsgrund mit irgendeinem Sinnesorgan sogenannter höherer Tiere besitzen. Dementsprechend ist die Literatur dieser Frage, sowie die darüber geäusserten Ansichten, Vermutungen, Theorien, und Hypothesen sehr zahlreich. Wir können hier auf diese selbstverständlich nicht näher eingehen, und werden die Frage nur auf Grund der neuesten diesbezüglichen Literatur, hauptsächlich auf Grund der Untersuchungen von Buddenbrock (1919b), behandeln, weil eben diese Untersuchungen es nötig machen, die Frage hier überhaupt zu erörtern. Während nämlich die früheren Ansichten die Halteren meistens als Gleichgewichtsorgane oder Steuerorgane betrachteten, hat Buddenbrock mit seinen Untersuchungen die Vermutung nahe gelegt, dass die Halteren spezifische Stimulationsorgane seien.

Über die in Frage kommenden Organe genügt es zu bemerken, dass sie, als umgestaltete Hinterflügel der Dipteren kleine trommelschlägerförmigen, äusserst beweglichen Gebilde darstellen (Abb. 3), die während des Fluges und des Schwirrens um ihr Basalgelenk heftige Schwirrbewegungen ausführen. Im inneren der Halteren befinden sich verschiedene Sinnesorgane von chrodotonalem Typ. Die Einzelheiten dieser kennen wir hauptsächlich seit den eingehenden Untersuchungen von Pflugstädt (1912), die aber über die Funktion dieser Sinnesorgane gar keine sicheren Schlüsse zulassen.

Was über die Halteren experimentell am leichtesten festzustellen ist, hat man schon längst erkannt, nämlich dass nach Entfernung der Halteren die Dipteren ihr

normales Flugvermögen durchaus verlieren. Wie dies aber zustande kommt, wie die Halteren einen Einfluss auf das Flugvermögen ausüben, ist auch heute noch nicht einwandfrei nachgewiesen.

Buddenbrock hat schon (1917), ehe er sich noch experimentell mit der Frage beschäftigte, aus theoretischen Gründen die Vermutung geäussert, die Halteren seien Organe zur Erzeugung nervöser Erregungen. Er ist zu dieser damals völlig neuen und eigenartigen Auffassung durch Analogieschlüsse gekommen, und zwar ausgehend von dem Uexküllschen Versuch über die Randkörper der Medusen. Obzwar, wie wir gesehen haben, die Basis dieser Analogieschlüsse heute als unsicher erscheint, haben sie sich als eine äusserst fruchtbare Arbeitshypothese bewährt.

Als sich nämlich Buddenbrock die Gelegenheit bot seine Hypothese experimentell nachzuprüfen, hat er diese in vielen Punkten so weitgehend bestätigen können, dass man seine Auffassung heute als "die vermutliche Lösung der Halterenfrage" betrachten kann.
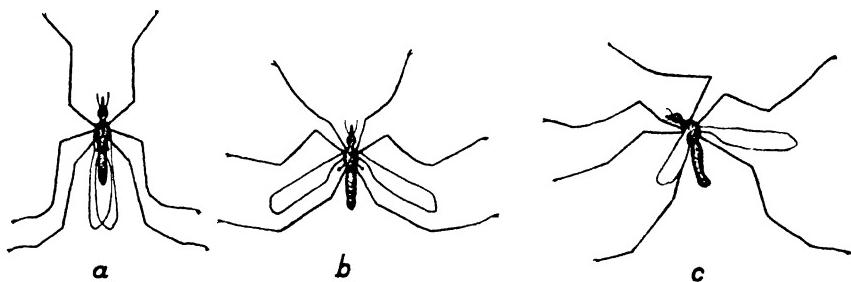
Abb. 4. Verschiedene Stellungen von *Tipula*, nach Buddenbrock. *a*, Ruhestellung; *b*, Bereitschaftstellung; *c*, Zwangsstellung nach Entfernung der Halteren.

Buddenbrock (1919*b*) hat zuerst nachgewiesen, und zwar hauptsächlich bei der Mücke *Tipula*, dass die Halteren echte tonuserzeugende Organe sind. Nach ihrer Ausschaltung treten verschiedene Zwangsstellungen ein (Abb. 4*c*), die eine sehr auffallende Ähnlichkeit mit echten Tonus-Ausfallerscheinungen haben, und als tonisch bedingt betrachtet werden können. Ein interessanter Versuch wurde mit der Fliege *Sarcophaga* durchgeführt. Diese konnte zur vollständigen Flügellähmung gebracht werden, wenn sowohl die Halteren als auch die Beine entfernt waren. Diese Erscheinung kann als ein Beweis dafür angesehen werden, dass die Halteren Organe zur Erzeugung nervöser Erregung sind, ebenso wie die Sinnesorgane der Beine, deren ähnliche Wirkung schon früher von Matula (1911) angenommen war. Entfernung der Halteren selbst kann die völlige Lähmung nicht hervorrufen, da ihre Wirkung durch die anderen in den Beinen befindlichen Stimulationsorgane einigermassen kompensiert wird, und ähnlich steht es mit der alleinigen Wirkung der letzteren. Wenn aber alle diese Organe, durch gleichzeitige Entfernung sowohl der Halteren als auch der Beine ausgeschaltet werden, so entsteht eine völlige Lähmung, bzw. Flugunfähigkeit. Bei anderen Fliegen war dies nicht der Fall gewesen, weshalb angenommen wird, dass bei diesen sich noch weitere

Erregungsorgane, und zwar aller Wahrscheinlichkeit nach in der Basis der Vorder-
flügel befinden.

Buddenbrock war auch bestrebt zur Verstärkung seiner eigenen Theorie die
Unhaltbarkeit der früheren Ansichten über die Funktion und Bedeutung der
Halteren nachzuweisen. Diese älteren Ansichten lassen sich dahin zusammenfassen,
dass "sie die mechanische Bewegung des Schwingers für das Massgebliche hal-
ten" (Buddenbrock, 1919*b*, p. 127). Danach wurden die Halteren bald als passiv
bewegliche Gleichgewichtsorgane, bald als aktiv wirkende Steuerorgane angesehen.
Gegen die vorige Auffassung wird hervorgehoben, dass die Halteren keine statischen
Sinnesorgane sein können, die ihre Lage passiv nach der Wirkungsrichtung der
Schwerkraft verändern, wie etwa die Statolithen der statischen Sinnesorgane, weil
sie sich aktiv bewegen, und einfach schon deshalb nicht als Gleichgewichtsorgane
in Betracht kommen können. Es wurde aber auch experimentell gezeigt, und zwar
an der Fliege *Eristalis tenax*, dass die Erscheinungen, die früher als Gleichgewichts-
störungen nach Halterenentfernung gedeutet wurden, auch durch Stutzen der
Flügel hervorgerufen werden können, wodurch doch ein Gleichgewichtsorgan
sicherlich nicht entfernt wurde.

Gegen die Steuerorganhypothese wird eingewendet, dass die Fliegen in die
Luft losgelassen auch nach Halterenentfernung ihren Abflug in bestimmter Rich-
tung steuern können, und zwar bei positiv phototaktischen Dipteren in der Rich-
tung der Lichtstrahlen. Dadurch wird jede weitere Diskussion über diese Frage
hinfällig, aber es wird auch noch gezeigt, dass das normale Abwärtsfliegen gar nicht
durch abwärts gerichtete Steuerungswirkung zustande kommt, sondern einfach
durch die Abnahme der Flugintensität (Verminderung der Frequenz, oder Kraft
der Flügelschläge), dass also die Halteren direkt nichts damit zu tun haben. Durch
den Schwirrflug wird der Körper immer emporgehoben, und nie nach dem Boden
gesteuert, sogar dann nicht wenn die Längsachse des Körpers kopfabwärts schräg
eingestellt ist. Die halterenlosen Fliegen können sich manchmal sogar noch vom
Boden emporheben, sinken aber bald wieder zurück, ihre Flugbahn unterscheidet
sich also nur durch ihre Ausmasse von der normalen. Ähnliche Ausfallerschei-
nungen können durch andersartige Verminderungen der Flugenergie, z.B. durch
einfaches Abstutzen der Flügel hervorgerufen werden. Die Halterenentfernung
beeinflusst also nur die Intensität und die Kraft des Fluges.

Die Steuerorganfunktion müsste ferner eine veränderliche Schwingungsrichtung
der Halteren zur Voraussetzung haben, die Beobachtungen an *Tipula gigantea*
zeigen aber, dass dies nicht der Fall ist, die Halteren schwingen bei jeder Körperlage
in derselben Richtung. Weinland (1891), der sich am eingehendsten mit der
Steuerungsfrage beschäftigt hat, kommt zwar zu der Überzeugung, dass die
Schwingungsrichtung der Halteren tatsächlich veränderlich ist, weshalb er sich auch
der Steuerorganhypothese anschliesst, seine Auffassung ist aber von histologischen
Befunde abgeleitet. Er fand nämlich an der Halterenbasis ein sehr bewegliches
Gelenk, dieser Befund kann aber gegen die Ergebnisse der unmittelbaren Beo-
bachtung nicht massgebend sein. Buddenbrock erklärt übrigens auch die Bedeu-
tung dieses beweglichen Basalgelenkes, indem er nachweist, dass sich die Halteren

in der Ruhestellung um dieses Gelenk gebeugt dicht an den Körper legen (Abb. 4a).

Endlich hat Buddenbrock mit Hilfe eines sog. Schusskymographions sehr überzeugende Beweise für die echten Folgen der Halterenentfernung geliefert. Das Schusskymographion bestand aus einer zwischen Schienen gleitenden vertikalen Platte, die mit berusstem Papier überzogen und mit Hilfe einer Feder auf den Schienen gleitend "abgeschossen" werden konnte. Die Flügelbewegungen einer dicht an der Platte aufmontierten Fliege wurden, auch wenn sie noch so heftig waren, am berussten Papier aufgezeichnet (vgl. Abb. 5). Mit dieser Einrichtung wurde gezeigt, dass die Halterenentfernung die Schlagfrequenz der Flügel bedeutend herabsetzt. Ein Flügelstummel, der beim Vorhandensein der Halteren ungefähr 240 Schwingungen pro Sek. machte, hat nach der Entfernung derselben nur etwa 190 Schwingungen in derselben Zeiteinheit ausgeführt (Abb. 5). Daraus könnte man zwar auf eine schlagfrequenzregulierende Wirkung der Halteren schliessen, was eine Bestätigung der Hypothese von Demoll (1918) wäre. Aber die weiteren Untersuchungen mit dem Schusskymograph haben gezeigt, dass eine solche reflektorische Regulierung, wie Demoll (1918) annimmt, nicht vorhanden ist. Diese Funktion könnte nämlich nur dann ausgeübt werden, wenn zwischen Flügel- und Halterenschlag ein gewisser Synchronismus bestünde. Dies ist aber durchaus nicht der Fall. Wenn man nämlich die Flügel stutzt, so erhöht sich die Schlagfrequenz derselben, während die Frequenz der Halterenschläge vollkommen unverändert bleibt. Es besteht also kein reflektorischer Zusammenhang zwischen der Schlagfrequenz der Flügel, bzw. Halteren, und die Beeinflussung der Flügelbewegungen durch die Halteren muss auf nichtreflektorischem Wege geschehen. So kommt Buddenbrock auf Grund aller seiner Befunde und Erörterungen zu der Auffassung, dass die Halteren echte Stimulationsorgane sind, die dem Nerven-, und dadurch dem Muskelsystem Erregungen (= Nervenenergie) zuführen. Diese können—teilweise eine Zeitlang gespeichert (wie z.B. die Erregungen der mechanischen Sinnesorgane der Tarsalglieder der
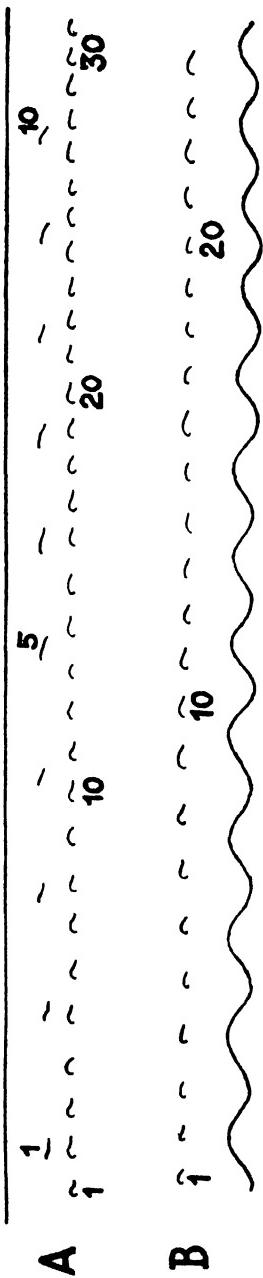

Abb. 5. Graphische Registrierung der Flügelschlagfrequenz mit Schusskymograph, nach Buddenbrock. A, Schlagfrequenz bei Vorhandensein von Halteren; B, dieselbe nach Entfernung der Halteren. Unten, Stimmgabelkurve von 80 Schwingungen pro Sek.

Beine während des Sitzens, wie Buddenbrock mit seinem Versuch an *Sarcophaga* nachgewiesen hat), teilweise unmittelbar nach ihrer Entstehung—zur Auslösung, bzw. Aufrechterhaltung normaler Flugreflexe verwendet werden.

Obwohl die Richtigkeit dieser Auffassung noch nicht ganz einwandfrei bewiesen ist, und wie erwähnt, die Betonung der Analogie der Halterenfunktion mit den Funktionen der Randkörper der Medusen die Überzeugungskraft der Buddenbrockschen Ausführungen nicht sehr günstig beeinflusst, muss man zugeben, dass die Auffassung von Buddenbrock noch immer eine so grosse Wahrscheinlichkeit besitzt, dass man kaum daran zweifeln kann, dass weitere Untersuchungen sie endgültig sicherstellen werden[1].

(c) *Die Tibialorgane von* Rhipipteryx chopardi.

Im Anschluss an die Besprechung der Halterenfrage sollen hier kurz noch die eigenartigen tibialen Sinnesorgane der Heuschrecke *Rhipipteryx chopardi* erwähnt werden, die Buddenbrock (1930) neuerdings auf Grund der Untersuchungen von Wille (1924) auch für spezifische Stimulationsorgane hält. Die Organe befinden sich an der Tibia der hinteren Extremitäten nahe zum Femur-Tibia Gelenk. Da die Hinterbeine am Gehen nicht beteiligt sind, wird die Tibia gewöhnlich in eine Rinne des Femurs eingeklappt gehalten, so dass das Tibialorgan völlig verborgen liegt. Nur beim Springen und während des Fluges werden die Hinterbeine derart ausgestreckt, dass die Organe mit der Aussenwelt in Berührung kommen.

Morphologisch ist das Organ nur wenig untersucht, gehört aber sicherlich zu den ziemlich einfachen Hautsinnesorganen. Von Wille wird es mit modifizierten Sinneshaaren der Antennen der Honigbiene verglichen. Es besteht aus einer Reihe von Zäpfchen, die in das Chitin der Ventralseite der Tibia eingesenkt sind, und nur mit ihrer Spitze die Oberfläche erreichen. Ferner sind in der Nähe dieser Zäpfchen zwei Sinnesgruben, bzw. Poren vorhanden, von denen die eine von aussen durch eine Lamelle verschlossen ist.

Über die Funktion führt Wille zuerst theoretische Erwägungen aus, und sucht nachzuweisen, dass die Organe Sinnesorgane, und nicht etwa Stridulationsorgane, Drüsen, oder dergl. sein können. Sie werden als Hautsinnesorgane betrachtet, die zur Wahrnehmung des Luftwiderstandes während des Springens oder Fluges dienen sollen, also eine Art Steuerorgan, Gleichgewichtsorgan, oder Organ zur Orientierung nach der Windrichtung darstellen sollen. Diese Auffassung wird durch eine Reihe schöner Versuche unterstützt. In einer Versuchsserie wurden Hinterbeine amputiert, und zwar sowohl unterhalb, also oberhalb des Tibialorgans, ferner einseitig, bzw. doppelseitig. In einer weiteren Serie wurden die Organe von aussen mit Kollodium verklebt. Nun wurde nachgewiesen, dass während normale Tiere sich an warmen sonnigen Tagen meistens durch grosse Sprünge und Gleitflüge fortbewegen, und diese Fähigkeit grösstenteils auch dann behalten, wenn das distale Ende der Tibia ein- oder beidseitig amputiert ist, können Tiere, deren Tibialorgane verklebt sind, obwohl die Beine übrigens vollkommen heil sind, nicht mehr

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[1] Siehe Nachträge, 2, Seite 417.

fliegen, und auch keine normalen Sprünge ausführen. Tiere deren Tibialorgan zur Kontrolle mit Wasser betupft wurde, flogen ungestört herum. Es ist bemerkenswert, dass nach Verkleben der Organe die normalen Flugbewegungen auch dann ausbleiben, wenn die Tiere in die Luft geworfen wurden, und hauptsächlich war ihre Landung sehr unsicher. Ferner wurde festgestellt, dass ein einseitiges Verkleben das Flugvermögen weniger beeinflusst als ein beidseitiges.

Wille sieht in diesen Versuchsergebnissen eine Bestätigung seiner obenerwähnten Auffassung, aber die Deutung von Buddenbrock (1930) scheint doch viel überzeugender zu sein. Man kann nicht einsehen, weshalb die Flugbewegungen in der Abwesenheit eines Gleichgewichtsorgans überhaupt nicht ausgeführt, und wie Wille erwähnt, in manchen Fällen die Flügel gar nicht entfaltet werden. Die Tiere dürften erst später durch die üblen Folgen der Ausschaltung dressiert werden. (Vgl. hierüber die Versuche Ewalds (1892 a) mit labyrinthektomierten Hunden.) Da dies nicht der Fall ist, da ferner in vielen Fällen die Bewegungen nur in ihren Ausmassen von den normalen abweichen (Länge der Gleitflüge maximal bis zu 5 m., gegen 10 m. bei normalen), muss man der Buddenbrockschen Auffassung eine grössere Wahrscheinlichkeit zuschreiben. Allerdings wären auch in dieser Frage weitere Untersuchungen noch höchst erwünscht.

(2) Die Stimulationswirkung der statischen Sinnesorgane.

A. *Das Labyrinth der Wirbeltiere als echtes " Tonusorgan."*

In dem vorangehenden Abschnitt haben wir gesehen, dass unter anderen auch jene Sinnesorgane, die zur Wahrnehmung der Lage und Bewegungen einzelner Körperteile dienen, eine bedeutende Stimulationswirkung ausüben. Noch viel bedeutender und wichtiger sind aber in dieser Hinsicht diejenigen Sinnesorgane, die zur Perzeption der Lage und Bewegungen des ganzen Körpers dienen, also die Organe zur Erhaltung des Körpergleichgewichtes (d.h. des sog. labilen Gleichgewichtes), die statischen Sinnesorgane. Man kann sogar sagen, dass diese Organe, wo sie überhaupt vorhanden sind, die Wirkungen des stimulatorischen Systems vollkommen dominieren. Die Stimulationswirkung dieser Organe ist vorwiegend eine Tonuserzeugung. Daher werden sie mit gewissem Recht als "Tonusorgane" bezeichnet. Daneben kommen aber auch kinetische Wirkungen vor, wie an betreffenden Stellen bemerkt wird.

Abweichend von der allgemeinen Regel, zuerst die primitiveren Systeme zu behandeln, wenden wir uns zuerst den statischen Sinnesorganen der Wirbeltiere zu, und zwar deshalb, weil bei ihnen der Einfluss auf den allgemeinen Muskeltonus erstmalig nachgewiesen wurde, und weil die diesbezüglichen Ergebnisse so allgemein anerkannt sind, dass man die späteren Befunde bei verschiedenen Wirbellosen einfach nur an diese anzuknüpfen braucht.

Abgesehen von den früheren, in einigen Punkten schon sehr klaren Kenntnissen über tonisierende Wirkungen des "inneren Ohres," hat zuerst J. R. Ewald (1892 a) durch seine sehr umfangreichen vergleichenden Studien die allgemeine tonisierende Wirkung gewisser Labyrinthteile erkannt. Seine Untersuchungen haben gezeigt,

dass eine operative Entfernung beider Labyrinthe bei Hunden eine allgemeine Muskelschwäche und Schlaffheit verursacht. Der Kopf schwankt, wird tiefer getragen als gewöhnlich, die Füsse der Tiere rutschen sehr oft aus, und ein Marschieren auf Hinter- oder Vorderbeinen ist sehr schwierig. Das Schlucken und Kauen ist auch sehr behindert, obwohl ein offensichtliches Nahrungsbedürfnis vorliegt. Das Maul lässt sich leicht öffnen, den Unterkiefer kann man widerstandlos bewegen. Nach mehreren Tagen verschwinden die meisten Ausfallerscheinungen, was man heute hauptsächlich auf eine kompensatorische Wirkung anderer tonisierender Organe (z.B. Reflextonus (vgl. S. 377) und hauptsächlich Lichttonus) zurückführen kann. Einige interessante Ausfallerscheinungen bleiben aber weiterhin bestehen. Schon vor den Untersuchungen von Ewald hat Schiff (1891) nachgewiesen, dass beiderseits labyrinthektomierte Hunde vom Tisch nicht herabspringen und eine Treppe nicht herabgehen. Ewald hat nun diesbezüglich festgestellt, dass zuerst diese Scheuheit nicht besteht, die Tiere springen nach Labyrinthentfernung normalerweise herab, schlagen sich aber sehr heftig an, und hauptsächlich ihr Kopf schlägt wie eine leblose Masse auf. Es besteht also eine allgemeine Muskelschlaffheit, Ungefedertheit, und das Tier vermeidet das Herabspringen, u.s.w., weil es durch mehrmalige misslungene Versuche und durch die üblen Folgen derselben dressiert wird. Auch Kauschwäche besteht noch nach Tagen, und Hunde, die früher Knochen gierig angenommen haben, können diese nicht mehr fressen.

Ewald hat auch mit Vögeln, hauptsächlich mit Tauben, Versuche angestellt, von welchen schon Flourens (1824) nachgewiesen hat, dass sie ihr Flugvermögen nach Labyrinthentfernung verlieren. Eine beiderseits labyrinthektomierte Taube kann den Kopf nicht ohne Schwierigkeit aufrecht halten, und wenn der Kopf nach hinten gebeugt nur mit einem ganz kleinen Gewicht belastet wird, bleibt die Taube stundenlang in dieser unnatürlichen Lage. Noch überzeugender ist der Versuch, wenn einer labyrinthektomierten Taube die Augen verbunden werden. Da diese jetzt sich weder mit dem Gleichgewichtsorgan, noch durch optische Fixierungen orientieren kann, sinkt der Kopf allmählich wie leblos nach hinten (Abb. 6). Auch die Atembewegungen verändern sich. Ihre Frequenz nimmt ab, aber die Atemzüge werden tiefer. Dies ist aber vielmehr eine kinetische Ausfallerscheinung der Labyrinthektomie.

Bei einseitiger Labyrinthexstirpation zeigt sich ein einseitiger Tonusverlust, obwohl sich einige Wirkungen der Labyrinthe auf beiden Körperseiten erstrecken, und antagonistische Muskeln (z.B. Strecker und Beuger einer Extremität) von je einem anderen Labyrinth beeinflusst werden. Diesbezüglich waren Frösche besonders günstige Versuchsobjekte. Die Tiere biegen nach einseitiger Labyrinthentfernung ihren Rumpf und den Kopf nach der operierten Seite offenbar wegen Tonusverlust der diesseitigen Muskeln des Rumpfes und Halses, während die Beine an dieser Seite adduziert, an der entgegengesetzten Seite gestreckt werden (Abb. 7). Im Wasser halten sich die Frösche nach einseitiger Labyrinthektomie ganz schief, wenn man aber die Lungensäcke ansticht, wird die Haltung etwas normaler. Also auch hier ist die Muskulatur der Atembewegungen mit beeinflusst. Dies wurde später auch zahlenmässig nachgewiesen (vgl. Graham-Brown, 1909). Wenn Tiere sich nach einseitiger Labyrinthexstirpation bewegen, entstehen Kreisbewegungen, wie z.B. bei Fröschen im

Wasser oder frei aufgehängten Tauben. Es ist aber nicht sicher, ob diese echte Tonus-Ausfallerscheinungen darstellen. (Vgl. hierüber die Ausführungen über Lichttonus.)

Weitere Versuche (auch mit Kaninchen, Kakadus, Salamandern, u.s.w.) haben noch weitere Einzelheiten ergeben, hauptsächlich betreffs der Frage, in welchem Grade die einzelnen Muskelgruppen von den Labyrinthen beeinflusst werden. Es hat sich herausgestellt, dass je präziser ein Muskel normalerweise seine Bewegungen ausführt, umso ausgeprägter die Folgen der Labyrinthentfernung sich daran zeigen. Deshalb werden Augen- und Halsmuskeln am meisten gestört, und es ist interessant, dass die Beinmuskulatur des Kakadus, deren Präzision in Greifbewegungen allbekannt ist, nach Labyrinthentfernung viel mehr Ausfallerscheinungen zeigt, als die in dieser Hinsicht weniger präzise Beinmuskulatur der Taube. Die weitere
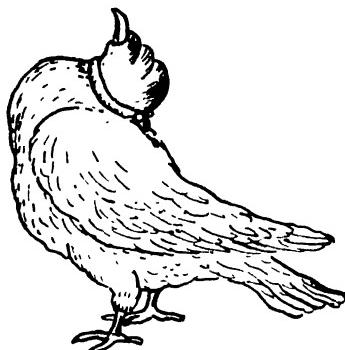
Abb. 6.
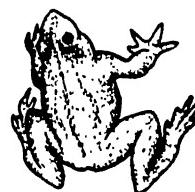
Abb. 7.

Abb. 6. Doppelseitig labyrinthektomierte Taube, mit verbundenen Augen, nach J. R. Ewald. Der Kopf kraftlos nach hinten gesunken.

Abb. 7. Linksseitig labyrinthektomierter Frosch, nach J. R. Ewald. Der Kopf und Körper nach links verdreht, die Extremitäten links adduziert, und flektiert, rechts abduziert, und extendiert.

Analyse dieser Erscheinungen führt aber schon zur Kenntniss der sog. tonischen Lagereflexe, welche heute eine sehr umfangreiche Literatur besitzen (vgl. Fischer, 1926; Magnus und de Kleijn, 1926 a u. b), die aber keine stimulatorischen Funktionen des Labyrinthes sind. Wie nämlich Buddenbrock (1924-28) ausführt, beziehen sich diese nur auf bestimmte Reflexbahnen, und sind ausgesprochene Reflexe. Sie unterscheiden sich von den gewöhnlichen Reflexen nur darin, dass ihre Wirkung sich nicht durch Muskelkontraktion, sondern in Tonusänderung der betreffenden Muskeln äussert. Allerdings besteht äusserlich keine scharfe Grenze zwischen der allgemeinen Stimulationswirkung und den tonischen Reflexen.

Die Lehre von Ewald, dass ein Teil des Labyrinthes, das sog. "Tonuslabyrinth," eine allgemeine tonisierende Funktion auf das gesamte Nervenmuskelsystem ausübt, wurde im Laufe der späteren Jahren durch eine grosse Fülle von Tatsachen bestätigt, bzw. erweitert. Dabei wurde aber die Aufmerksamkeit von den allgemeinen Stimulationserscheinungen, zugunsten der tonischen Lagereflexe ziemlich

abgelenkt. Die Lehre hat aber auch so noch eine sehr umfangreiche Literatur, von welcher nur folgende Punkte hervorgehoben werden sollen.

Die Kraftabnahme der Muskeln nach Labyrinthexstirpation wurde bald auch quantitativ festgestellt (Ewald, 1892 b, 1894; Emanuel, 1903; Bickel, 1903; und am exaktesten Benjamins u. Huizinga, 1928). Ferner wurde der Einfluss der Labyrinth-exstirpation auf die Geschwindigkeit des Eintretens der Totenstarre nachgewiesen, und festgestellt, dass die sog. Nystensche Reihe des Eintretens der Totenstarre in den einzelnen Muskeln mit derselben Reihenfolge übereinstimmt, in welcher die Muskeln vom Labyrinth beeinflusst werden (Ewald u. Willgerodt, 1896). Auch durch klinische Fälle war die Lehre von Ewald bestätigt worden (vgl. Allers, 1909), wobei nach verschiedenen Erkrankungen des Tonuslabyrinthes ein allge-meiner Tonusverlust, resp. eine Zunahme konstatiert wurde. Die neueste Proble-matik und Literatur der Lehre findet man bei Huizinga (1933).

Die weiteren Untersuchungen über die allgemeine Stimulationsfunktion des Labyrinthes beziehen sich auf die Erweiterung des Gültigkeitsbereichs der Ewald-schen Feststellungen auf vergleichend physiologischem Gebiete. An Reptilien wurden diesbezügliche Untersuchungen u.a. von Henri (1899), ferner von Trende-lenburg u. Kühn (1908), ausgeführt. Sowohl bei Schlangen (z.B. Ringelnatter), wie bei Eidechsen und Schildkröten wurde neben verschiedenen Lagereflexen, u.s.w. auch ein allgemeiner Tonusverlust nach Labyrinthexstirpation nachgewiesen. Als Hauptzeichen dieses Verlustes zeigt sich in den Versuchen die Neigung des Kopfes und Rumpfes nach einseitiger Labyrinthentfernung nach der operierten Seite. Ferner wird aber auch eine allgemeine Abschwächung und Verlangsamung der Bewegungen nach doppelseitiger Labyrinthektomie festgestellt.

Bei Amphibien, und zwar bei Fröschen, haben neben den schon erwähnten Untersuchungen weitere, wie z.B. diese von Wlassak (1892), vom Gesichtspunkte des Stimulationsproblems nicht viel neues erbracht.

Bei Fischen wurde eine ganze Anzahl Untersuchungen ausgeführt, teilweise mit der Absicht, für die Loebsche Tropismenlehre, die ja mit dem Problem der Tonus-erzeugung innig verbunden ist, weitere Argumente zu verschaffen. Eben deshalb können wir nicht auf alle Einzelheiten der diesbezüglichen Literatur eingehen.

Es ist auch bei Fischen allgemein, dass nach einseitiger Labyrinthexstirpation der Körper nach der operierten Seite geneigt wird. Ferner treten verschiedene Zwangstellungen und Zwangsbewegungen auf. Bethe (1894) hat bei Süsswasser-fischen (*Perca, Scardinius*) bis 70° reichende Neigung beobachtet, wozu er bemerkt, dass die volle Erscheinung erst später eintritt, und durch Anstechen der Schwimm-blase etwas korrigiert werden kann (vgl. Ewald, 1892 a). Loeb (1891 a, b) hat bei Haifischen 20–50° Neigung der Körperachse nach der Operationsseite festgestellt. Die Zwangsstellungen bestehen meistens im Verdrehen des Kopfes, seitwärts, oder aufwärts, Krümmung des Rumpfes, oder des Schwanzes, u.s.w. Solche Erschei-nungen wurden öfters bei Haifischen (Lee, 1894; Loeb, 1891 b; Gaglio, 1903, etc.), ferner beim Flussaal (W. F. Ewald, 1907), Seepferdchen (Fröhlich, 1940 c), u.s.w. beobachtet. Die Zwangsbewegungen: Kreisbewegungen um die Längsachse, oder nach der einen Seite ("Manégebewegungen") sind vielleicht nicht auf Tonus-

wirkungen zurückzuführen, wie dies oft angenommen wurde, sondern auf gewisse kinetische Effekte der Labyrinthexstirpation. Die von Bethe (1894) beobachtete Kraftabnahme der Atembewegungen (Kiemendeckelbewegungen) an der operierten Seite ist eine ähnliche Ausfallerscheinung. Dagegen können die Zwangshaltungen als echte Tonus-Ausfallerscheinungen betrachtet werden.

Eine solche noch allgemeinere Erscheinung ist die Herabsetzung der Muskelkraft, hauptsächlich nach beidseitiger Labyrinthentfernung. Dies wurde von Bethe (1894) beim Barsch (*Perca*) und beim Rotauge (*Scardinius*) festgestellt, und Gaglio (1903) und W. F. Ewald (1907) haben auch zahlenmässige Messungen darüber ausgeführt. Der Kraftverlust soll nach Gaglio (1903) bei Haifische ungefähr 50 % sein, bei Aalen nach Ewald (1907) annähernd 30 %.

Es soll hier bemerkt werden, dass der neuestens erbrachte Nachweis, dass das Labyrinth der Fische als Gehörorgan funktioniert (v. Frisch u. Stetter, 1932), den geschilderten Beobachtungen und Versuchsergebnissen nicht widerspricht, da die Stimulationswirkungen immer nur als Nebenfunktionen des inneren Ohres betrachtet wurden.

B. *Die Stimulationswirkung der Statocysten der Wirbellosen.*

Bald nach den ersten Ergebnissen der Untersuchungen über Labyrinthtonus wurden ähnliche Erscheinungen bei den Gleichgewichtsorganen der Wirbellosen beobachtet. Bei verschiedenen Klassen und Ordnungen der wirbellosen Tiere wurde prinzipiell dasselbe festgestellt, was vom Labyrinth der Wirbeltiere bereits schon bekannt war.

Bei Würmern hat die Tonusfunktion der Statocysten v. Buddenbrock (1912, 1913) bei der Untersuchung der geotaktischen Erscheinungen nachgewiesen. Zuerst haben seine Untersuchungen bei *Arenicola* (und gleichzeitig bei der Holothurie *Synapta*) nur sog. tonische Lagereflexe nachgewiesen, ähnlich denen, wie sie von Magnus und de Kleijn (1913 *a* und *b*) eben um diese Zeit beschrieben wurden. Dagegen wurden bei *Myxicola*, und noch klarer bei *Branchiomma*, zwei im Meeressand grabenden Polychaeten, echte tonuserzeugende Dauerwirkungen nachgewiesen, die von den Statocysten ausgehen. Bei *Myxicola* liess sich zeigen, dass bei Tieren, deren Statocysten entfernt wurden, die Bewegungen des Schwanzteiles geschwächt waren. Da bei diesen Tieren die Entfernung der Statocysten nur in Narkose möglich ist, wurden normale Tiere zur Kontrolle auch narkotisiert. Wenn man nun solche neben operierte Tiere legt und die Schwanzteile aller mit Sand bedeckt, so werden die nichtoperierten Exemplare Bewegungen mit dem Schwanze ausführen, um diesen vom Sand zu befreien, während die operierten gar keine solchen Bewegungen zeigen, da ihnen das Heben des Schwanzes offenbar sehr schwer fällt. *Branchiomma* ist ein günstigeres Objekt für solche Untersuchungen, da es die Statocystenentfernung ohne Narkose aushält. Hier wurden also die Folgen der Operation weiter analysiert, und festgestellt, dass nach Entfernung der Statocysten eine allgemeine Verminderung des Muskeltonus eintritt. Eine ähnliche Erscheinung kann auch bei Durchtrennung des Bauchmarkes dicht hinter dem Kopfe erzielt werden. Diese Operation hat aber einen noch grösseren Tonusverlust

zur Folge da dadurch nicht nur die Wirkung der Statocysten, sondern die der vordersten Ganglien zusammen ausgeschaltet wird. Der Tonusverlust äussert sich übrigens meistens darin, dass die Tiere, die sich normalerweise sofort in den Sand eingraben, nach Statocystenentfernung manchmal mehrere Stunden zu dieser Funktion brauchen. Es sind übrigens bei *Branchiomma* auch tonische Lagereflexe wieder nachgewiesen und ein allgemeiner Muskelsinn beobachtet worden. Ferner wurde festgestellt, dass einseitige Statocystenexstirpation keine Folgen hat, dass also beide Statocysten offenbar den ganzen Körper mit Dauererregungen versehen können. Die biologische Wichtigkeit der statischen Stimulationsfunktion wurde von Buddenbrock durch die Beobachtung jener Erscheinung gezeigt, dass Arten, die keine Statocysten haben (z.B. *Sabella pavonina*), die Einbohrbewegungen viel langsamer ausführen, und es oft tagelang dauert bis sie sich verkriechen.

Unter den Weichtieren wurde die Stimulationsfunktion der Statocysten von Tschachotin (1908) bei der Heteropode *Pterotrachea* nachgewiesen, wobei auch die anatomischen Verhältnisse eingehend untersucht wurden. Auf die Beschreibung der komplizierten Struktur der Statocysten können wir nicht eingehen, es soll nur bemerkt werden, dass im Lumen der blasenförmigen Statocyste neben Sinneszellen noch grosse Zellen mit Wimperflammen vorhanden sind. Die Wimperflammen bewegen sich rhythmisch und hierdurch wird das Statolith periodisch in Bewegung gesetzt. Da haben wir also ein Beispiel für rhythmische Dauerreize vor uns, deren Rolle mit der alleinigen reflektorischen statischen Funktion des Organs nicht erklärt werden kann.—Die Untersuchungen von Tschachotin stellten fest, dass das Zentrum für Muskeltonus im Zerebralganglion liegt. Der Zerebraltonus kann beim Durchschneiden der Kommissuren zwischen Zerebral- und Pedalganglion nachgewiesen werden, indem eine solche Ausschaltung des Zerebralganglions zu schweren Bewegungsanomalien (Umkippen, Schwimmen in verkehrter Lage, u.s.w.) führt, die auf Tonusverlust zurückgeführt werden. Ob dies mit vollem Recht geschieht, soll dahingestellt bleiben. Jedenfalls findet man nach Statocystenentfernung ähnliche, obzwar nicht so ausgeprägte Anomalien, und wenigstens einige davon sind sicherlich Folgen eines Stimulationsausfalles. Die Stimulationsfunktion der Statocysten bezieht sich übrigens auf dieselbe Körperseite, wo sich die Statocyste befindet, da nach einseitiger Exstirpation die Muskulatur an der Operationsseite erschlafft. Die Wirkung erstreckt sich jedoch auf beide Körperseiten, was auch anatomisch bewiesen wurde, indem sich die Nervenfasern im Zerebralganglion teilweise überkreuzen.—Diese Befunde wurden später von Polimanti (1911) bestätigt, indem er nachwies, dass nach der Ausschaltung einer Statocyste durch örtliche Kokainnarkose, der Muskeltonus beiderseits sinkt, aber an der einen Seite geschieht dies in höherem Masse. Merkwürdigerweise waren bei seinen Versuchen die Muskeln der kontralateralen Seite mehr erschlafft.

Bei Cephalopoden ist die Stimulationswirkung der Statocysten auch schon seit langem bekannt. Schon Delage (1886, 1887) hat das Auftreten von Bewegungsstörungen an *Octopus* nach Statocystenentfernung beobachtet, diese wurden aber durch Exstirpation der Augen aufgehoben. Uexküll (1894) hat an *Eledone moschata* ähnliches festgestellt. Die Erscheinungen wurden von Fröhlich (1904a) ebenfalls

an *Eledone* eingehender studiert, und in Übereinstimmung mit den früheren Ansichten von Muskens (1904) wurden als Ursache der Bewegungsstörungen die Veränderungen des Muskeltonus bezeichnet. Muskens führt als Beweis für diese Auffassung den erschlafften Zustand der Muskulatur und auffallende Bewegungslosigkeit der Tiere nach Statocystenexstirpation an. Fröhlich konnte nun diese Erscheinungen auch graphisch registrieren, und ausser der Erscheinung, dass nach "Statocystenläsion"[1] die Kraft der Saugnäpfe fast völlig verloren geht, stellte er die Zunahme der Dehnbarkeit der Muskeln quantitativ fest (Abb. 8). Auch die Frequenz der Atembewegungen ist nach Statocystenläsion vermindert, was auf eine kinetische Stimulationswirkung der Gleichgewichtsorgane schliessen lässt.

Neben diesen Beiträgen zur Kenntnis der Stimulationsfunktion der Statocysten hat Fröhlich (1904 b) ähnlich wertvolle Untersuchungen über dieselbe Funktion bei Crustaceen ausgeführt. Auch hierüber waren schon frühere Angaben von Delage
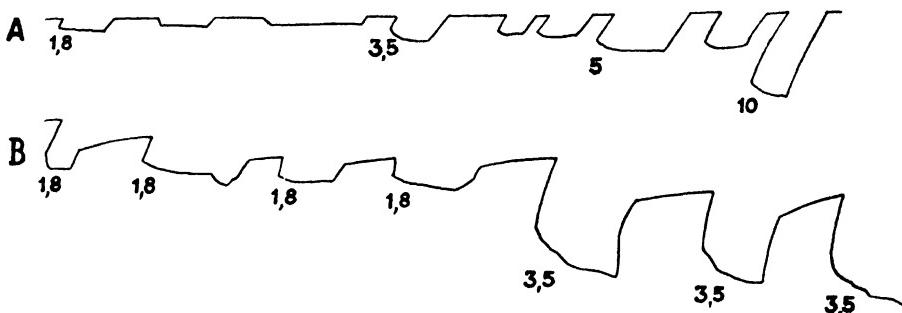

Abb. 8. Dehnungskurve der Muskulatur von *Eledone moschata*, nach Fröhlich. *A*, normales Tier; *B*, Tier, dessen Statocysten vorher entfernt wurden. Die Nummern geben die Belastung in Gramm an.

(1887) über *Palaemon*, *Gebia*, *Corystes*, *Polybius*, u.a. vorhanden, nach welchen eine Statocystenexstirpation verschiedene Bewegungsstörungen hervorrief, es war aber nicht näher analysiert, wie weit diese auf Gleichgewichtsstörungen, bzw. Tonusverlust zurückzuführen sind. Auch Beer (1898–99) hatte Untersuchungen über die Folgen der Statocystenentfernung (nach seinem unschönen Fachausdruck: "Entstatung") angestellt, diese waren aber ziemlich einseitig eingestellt, da sie grösstenteils zur Entscheidung einer Diskussion über den angeblichen "Gehörsinn" der Krebse (vgl. Hensen, 1899) dienen sollten. Sie liessen jedoch auch auf eine Tonusfunktion der Statocysten schliessen.

Die Untersuchungen von Fröhlich (1904 b) an *Penaeus* haben nun festgestellt, dass die typischen Rollungen der Crustaceen nach Statocystenläsion auf eine Schwächung der Muskulatur an der kontralateralen (d.h. der operierten entgegengesetzten) Seite beruhen. Ähnliche Rollungen konnten auch dann hervorgerufen werden, wenn die Beine an der einen Seite andersartig, z.B. durch Zusammenbinden, an ihren normalen Bewegungen verhindert wurden. Wenn diese Versuche auch nicht ganz klar machen, ob es sich hier um tonische oder kinetische Ausfall-

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[1] Dieser Ausdruck für die Operation wurde von ihm eingeführt.

erscheinungen handelt, haben die weiteren Untersuchungen über Muskelelastizität bei passiver Dehnung einen ausgesprochenen Tonusverlust nach Statocystenentfernung nachgewiesen. Das Tier wurde fixiert und die Dehnbarkeit der Schwanzmuskulatur, "die bei *Penaeus* den weitaus überwiegenden Teil der Gesamtmuskulatur ausmacht," durch verschiedene Belastung eines damit verbundenen Hebels auf ein Kymographion registriert. Die Ergebnisse zeigen, dass nach Statocystenläsion dasselbe Gewicht eine grössere Dehnung verursacht als vorher. Die Exstirpation wurde übrigens einfach durch Auskratzen oder Auswaschen der Statolithen vollbracht.

Diese Befunde stehen mit den Angaben von Bethe (1897) über *Carcinus maenas* in vollem Einklang. Von den verschiedenen Versuchen Bethes an dieser Krabbe soll nur die Messung der Muskelkraft der Extremitäten hervorgehoben werden, die ebenfalls eine Schwächung der Muskulatur nach Entfernung der Statocysten ergab.

Die weiteren Untersuchungen auf diesem Gebiete beziehen sich hauptsächlich auf die Springreflexe der Mysideen. Bekanntlich führen diese Krebse als Fluchtreaktionen mächtige Schläge mit ihrem Schwanz nach unten aus, wodurch sie grosse, sogar das 20-fache ihrer Körperlänge messende Sprünge machen können. Diese Reaktionen dienen als ausgezeichneter Test für die Untersuchung des Einflusses der Statocysten auf die Muskelkraft. Schon die erwähnten früheren Untersuchungen von Delage (1887), Hensen (1899), und Beer (1898–9) haben hierüber berichtet, viel überzeugender waren aber die Untersuchungen von Bethe (1895) in dieser Hinsicht. Es konnte nämlich gezeigt werden, dass nach Entfernung der Statocysten der Schwanz eine typische Zwangshaltung einnimmt, und zwar stark aufwärts gebogen wird. Dies muss auf eine Erschlaffung der normalerweise überwiegenden ventralen Flexormuskulatur zurückgeführt werden, es ergibt sich also ein ausgesprochener Tonusverlust nach Labyrinthexstirpation. Bauer (1908) hat nun von diesen Tatsachen ausgehend die alleinige Bedeutung der Schwanzmuskulatur beim Zustandekommen der verschiedenen Bewegungsstörungen betont. Seine Versuche haben bei *Macropsis Slabberi* ergeben, dass die Entfernung der Statocysten eine ähnliche Aufwärtskrümmung des Abdomens verursacht, wie es bereits Bethe (1895) beschrieben hat, die anderen Bewegungsorgane verbleiben aber normal. Danach bezieht sich also die Stimulationswirkung der Statocysten bei den Mysideen ausschliesslich auf die Schwanzmuskulatur, und die von den verschiedenen Autoren beschriebenen Bewegungsstörungen nach Statocystenentfernung sollen nicht auf Störungen in der Beinmuskulatur, sondern ausschliesslich auf solche in der Schwanzmuskulatur zurückgeführt werden. Dies steht offenbar etwas in Widerspruch mit den von Bethe (1897) an *Carcinus*, und von Fröhlich (1904 *b*) an *Penaeus* erzielten Ergebnissen, obwohl die überwiegende Bedeutung der Schwanzmuskulatur bei den Bewegungen, bzw. deren Störungen auch von diesen Forschern schon betont wurde. Es ist aber möglich, dass die Mysideen eine Sonderstellung einnehmen, und bei ihnen eben wegen der erwähnten spezifischen Springreflexe die Stimulationsfunktion der Statocysten eine spezifische Modifikation erleidet.

Allerdings geht aber aus allen geschilderten Untersuchungen die Tatsache hervor, dass die Statocysten der Wirbellosen, ebenso wie die Gleichgewichtsorgane der Wirbeltiere eine hervorragende stimulatorische Funktion im Organismus ausüben.

(3) Die Stimulationsfunktion der Lichtsinnesorgane.

A. *Lichttonus und verwandte Erscheinungen als Stimulationswirkungen der Lichtsinnesorgane.*

(a) *Bei Wirbellosen.*

Neben den spezifischen "Tonusorganen," wie man oft die statischen Sinnesorgane bezeichnet, kommt noch den Lichtsinnesorganen eine ganz hervorragende, und der statischen Stimulation in jeder Hinsicht gleichstellbare tonisierende Stimulationsfunktion zu, welche zur Unterscheidung von der vorigen als Lichttonus oder Phototonus bezeichnet wird. Von dem Lichttonus können ebenso wie von dem Labyrinthtonus, bzw. Tonus der Gleichgewichtsorgane einige nichttonische Erscheinungen kaum abgegrenzt werden. Hier zeigt sich wieder wie schwer die von Bozler postulierte Auseinanderhaltung tonischer, bzw. kinetischer Stimulationen durchzuführen ist. Wir werden letztere grösstenteils mit den Erscheinungen des echten Lichttonus zusammen behandeln, und in einem nächsten Abschnitt werden nur einige spezielle photokinetische Erscheinungen, besonders im Zusammenhang mit dem Ocellenproblem, gesondert besprochen.

Ob die hier zu beschreibenden Erscheinungen stimulatorischer Natur sind, ist eigentlich einwandfrei noch nicht bewiesen. Einige können ebenso gut tonische Lagereflexe sein. Es gibt aber indirekte Beweise dafür, dass die meisten doch echte Stimulationswirkungen sind. Zuerst die Analogie dieser Erscheinungen zu echten Stimulationserscheinungen. Weiterhin die Dauernatur der Erregungen. Licht ist ja die allgemeinste Reizquelle, die dem Organismus sozusagen immer zur Verfügung steht. Endlich beziehen sich Lichttonuserscheinungen und verwandte Phaenomene meistens auf das Gesamtbewegungssystem, oder wenigstens auf grössere Muskelgruppen.

Erscheinungen des Lichttonus spielten allzeitig eine wichtige Rolle in den Diskussionen über die Loebsche Tropismenlehre, hauptsächlich bei den sog. "heliotropischen" Erscheinungen, welche, wie bekannt, ganz allgemein auf eine Lichttonuswirkung zurückgeführt werden sollten. Es würde zu weit führen die Einzelheiten dieser Polemiken hier zu erörtern, und wir werden die Ergebnisse der Untersuchungen über Lichttonus so weit wie möglich davon unabhängig darstellen und besprechen.

Schon seit langem, und aus verschiedenen Beobachtungen und Versuchen ist bekannt, dass den Augen neben ihrer Hauptfunktion auch solche Aufgaben zukommen, welche denen des Labyrinthes und der Statocysten sehr ähnlich sind. Dies ergab sich vor allem aus der Beobachtungen von Insekten auf einer rotierenden Scheibe. Loeb (1888) hat durch solche Versuche festgestellt, dass die von der Drehung der Scheibe bedingten Bewegungen der Hausfliege anders ausfallen, als

diejenigen der Wirbeltiere. Bei Wirbeltieren üben nämlich einzelne Körperteile (Augen, Kopf) und der ganze Körper Bewegungen gegen die Richtung der Rotation aus, während nach dem Aufhören der Drehung heftige Nachdrehungen in entgegengesetzter Richtung erfolgen. Bei der Fliege ist nun aber nur die erste Bewegungsform vorhanden: das Tier bewegt sich während der Drehung der Scheibe gegen die Richtung derselben, sobald aber die Drehung aufhört, bleibt es stehen, ohne die Nachdrehungen in entgegengesetzter Richtung auszuführen. Daraus wurde der Schluss gezogen, dass die Bewegungen, die bei Wirbeltieren durch den Gleichgewichtsapparat bestimmt werden, bei der Fliege durch andere Mechanismen, und zwar durch den Augen reguliert werden, dass diesen also eine dem Gleichgewichtssinne ähnliche Funktion zukommt. Die Feststellung von Lyon (1900a), dass bei Fliegen die von der Drehung bedingten Bewegungen nach Blendung der Augen völlig ausbleiben, führte einen neuen Beweis für die Auffassung dass dieser Mechanismus in den Augen zu suchen ist. Auch die Untersuchungen von Rádl (1903) haben die Richtigkeit dieser Auffassung bewiesen. Rádl hat nämlich bei seinen Untersuchungen mit der Drehscheibe festgestellt, dass die Bewegungen der Insekten (hauptsächlich Fliegen, wie *Laphria*, *Calliphora*, und Libellen wie *Gomphus*, u.s.w.) bei passiver Rotation dadurch entstehen, dass sie mit den Augen Objekte fixieren und sich zu diesen orientieren.

Die verschiedenen Feststellungen über Zwangsbewegungen ("forced movements"), und deren engen Zusammenhang mit den Augen bewiesen auch, dass die Augen eine Art "Lichtgleichgewichtsapparat" darstellen. Solche Erscheinungen, wie die Ausführung von Manégebewegungen nach einseitiger Blendung, die zuerst von Holmes (1901) an Amphipoden (*Hyalella*, *Orchestia*, *Talorchestia*, und *Gammarus*), ferner von Parker (1903) an Schmetterlingen, z.B. *Vanessa*, sowie von Rádl (1904) an der Fliege *Dexia* nachgewiesen wurden, fernerhin die passiven Augenbewegungen nach Belichtung bei einigen Copepoden (Rádl, 1904; W. F. Ewald, 1910), und anderen Krebsen (Lyon, 1900a), galten jeder Zeit als die wichtigsten Beweise für die Tropismenlehre, also für die tonisierende Wirkung der Lichtsinnesorgane. Rádl (1903) hat die Augen schon auf Grund solcher Tatsachen als echte Organe für Muskeltonus bezeichnet. Wie wir aber weiterhin sehen werden, war durch diese Versuche noch nicht einwandfrei bewiesen, ob dem "Lichtgleichgewichtsapparat," ebenso wie dem Gleichgewichtsapparat in engerem Sinne eine typische tonisierende Stimulationsfunktion zukommt.

Es fehlt jedoch auch an solchen Beobachtungen und Untersuchungen nicht, die direkt für einen Lichttonuseffekt sprechen, und die Augen als Stimulationsorgane für diesen Effekt verantwortlich machen. Abgesehen von Angaben, die vielleicht keine echten Tonuswirkungen betreffen, war Axenfeld (1899) der erste, der Tonusdifferenzen an ruhenden Tieren festgestellt hat, was hauptsächlich später sehr wichtig war, da während der heftigen Polemik über die Tropismenlehre viele Erscheinungen, die früher auch als Tonus bezeichnet wurden, als Muskelbewegungen in engerem Sinne (obzwar von Lichtreize verursacht) sich herausstellten. Axenfeld sah bei Fliegen, deren obere Augenhälften geschwärzt waren, dass sie ihren Kopf hochheben, dagegen nach Schwärzung der unteren Augenhälften diesen zu

Boden senken. Er hat auch die interessante Beobachtung gemacht, dass einseitig geblendete Locustiden und *Carabus*, wenn sie auf eine glatte Unterlage gesetzt und mit dieser zusammen nach der geblendeten Seite geneigt werden, leicht daran herab gleiten. Werden sie jedoch nach der intakten Seite geneigt, so können sie sich festhalten. Rádl (1903), dessen eingehende Untersuchungen schon öfters erwähnt wurden, hat festgestellt, dass die Imago der Fliege *Gomphus*, von der Seite belichtet, den Rücken seitlich zu den Lichtstrahlen neigt. Wenn Fliegen, wie *Laphria*, *Lestes*, u.a. an der einen Seite geblendet werden, drehen sie ihren Kopf nach der Seite des sehenden Auges. Holmes (1905) hat gezeigt, dass bei der Wasserwanze *Ranatra* die Schreitbeine bei Belichtung von vorne fast völlig gestreckt werden und der Körper ganz flach am Boden liegt. Im Falle einer Beleuchtung von hinten sind die Beine gebeugt, und das Tier nimmt eine hochgerichtete Stellung ein. Die Erscheinung ist noch überzeugender, wenn das Tier von der Seite beleuchtet wird. Dann werden an der belichteten Seite die Beine gebeugt, an der Gegenseite gestreckt, und auch das Abdomen wird etwas nach der belichteten Seite gebogen. Carpenter (1905) hat ähnliches an *Drosophila* festgestellt. Dass diese verschiedenen Zwangsstellungen von den Augen beherrscht werden, hat Holmes (1905) auch gezeigt, indem er verschiedene Stellungen von *Ranatra* hervorrufen konnte wenn die Augen oder einige Stellen derselben geblendet waren. Diesbezügliche Untersuchungen sind jedoch hauptsächlich mit dem Namen Garreys (1918) verbunden, der an verschiedenen Tieren sehr eingehende, aber leider etwas einseitig gedeutete Untersuchungen anstellte. Sein Hauptobjekt war die Raubfliege *Proctacanthus*, er hat aber auch andere Fliegen (*Drosophila*, *Eristalis*, *Tabanus*), ferner verschiedene Schmetterlinge (*Vanessa*, *Argynnis*) benutzt. Nach völliger Ausschaltung der Augen wurde ein sehr ausgesprochener Tonusverlust beobachtet. *Proctacanthus* fliegt unsicher, kraftlos, und fällt bei Landung oft auf den Rücken, zeigt Zwangsstellungen (Proboscis und Abdomen zum Boden gesenkt), u.s.w. Normale Fliegen lassen am berussten Kymographpapier nur die Spuren der Beine, geblendete ziehen auch mit dem Körper eine Spur. Auch Schmetterlinge zeigen eine allgemeine Kraftlosigkeit und Unsicherheit nach der Ausschaltung der Augen. Wenn nur das eine Auge von *Proctacanthus* geblendet ist, so entstehen sehr charakteristische Zwangsstellungen: die Beine sind an der geblendeten Seite stark gestreckt, an der Gegenseite gebeugt, der Körper hängt nach der sehenden Seite über, und der Kopf ist noch mehr dahin geneigt (Abb. 9 *b*, *f*). Eine Messung zeigte, dass die Körperhaltung mit 30–60° von der Vertikalebene abweichen kann (je nach der Intensität des Lichtes!), während der Kopf immer noch mehr, um 50–90° geneigt ist. Schmetterlinge zeigen dieselben Zwangsstellungen. Die interessanten Untersuchungen über die Wirkung partieller Augenblendung haben ergeben, dass nach Blendung der unteren Augenhälften der Körper vorne aufgerichtet wird, und hinten zum Boden sinkt (Opisthotonus) (Abb. 9 *d*), während bei Schwärzung der oberen Hälften eine genau entgegengesetzte Stellung (Emprosthotonus) eingenommen wird (Abb. 9 *e*). Man beachte, dass diese Befunde in diametralem Widerspruch mit den Angaben von Axenfeld (1899) stehen. Durch Schwärzung der oberen Augenhälfte an einer Seite, und der unteren an der anderen Seite wurden noch extremere Zwangsstellungen, als durch

Blendung ganzer Augen hervorgerufen, und zwar Biegung nach der Seite, wo die untere Augenhälfte geblendet ist (Abb. 9*c*).

Bei den meisten geschilderten Untersuchungen handelt es sich offenbar um eine Tonusdifferenz der Beinmuskulatur je nachdem, von welcher Richtung das Licht die Augen trifft, d.h. welche Augenelemente, und mit welcher Intensität, sie beleuchtet werden.

Die Auseinandersetzungen von Garrey leiden jedoch unter einer Art "Schönheitsfehler," welcher darin besteht, dass sie allzu sehr im Dienste der Tropismenlehre stehen. Man kann sich von dem Gedanken nicht befreien, dass die Versuche in dieser Richtung schon etwas präoccupiert ausgeführt waren, und die Befunde manchmal einseitig zugunsten der Tropismentheorie gedeutet sind. Dies bezieht sich hauptsächlich auf die Erwägungen über die verschiedenen Zwangsbewegungen (Kreisbewegungen nach einseitiger Augenblendung), die auch alle auf Tonuser-
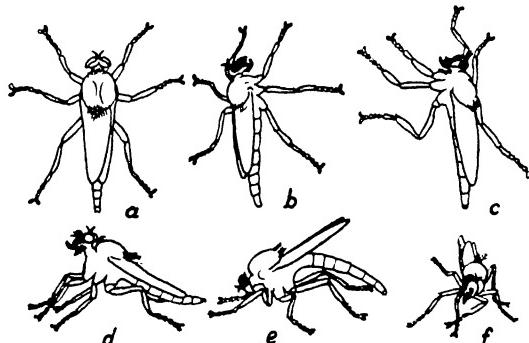

Abb. 9. Verschiedene Haltungen der Raubfliege *Proctacanthus*, nach Garrey. *a*, normal; *b*, rechtes Auge geblendet; *c*, am linken Auge die obere Hälfte, am rechten die untere Hälfte geblendet; *d*, beiderseits die untere Hälfte; *e*, beiderseits die obere Hälfte geblendet; *f*, wie in *b*, von vorne gesehen.

scheinungen zurückgeführt werden. Dies ist aber offenbar nicht richtig, da bei den verschiedenen phototaktischen Reaktionen auch typische Reflexe eine Rolle spielen.

Der scharfe Unterschied zwischen echten Tonuserscheinungen und phototaktischen Reaktionen wurde von Buddenbrock (1919*a*), fast gleichzeitig mit den Untersuchungen und Ausführungen von Garrey, nachgewiesen. Buddenbrock arbeitete mit Landschnecken (*Helix*), und hat zuerst die Tonuswirkung der Augen auch bei diesen Tieren festgestellt. Wie er erwähnt, hat schon viel früher Bohn (1904*a*, *b*) diese Tonuswirkung bei Schnecken erkannt. Bohn fand bei der Uferschnecke *Littorina*, dass sie von einem parallel zur Bewegungsrichtung aufgestellten weissen Schirm "abgestossen," von einem schwarzen "angezogen" wird. Dasselbe hat Buddenbrock bei Landschnecken festgestellt. Dies ist, wie er ausführt, eine echte Tonuswirkung, da der weisse Schirm als Lichtquelle funktioniert, und die "Abstossung," bzw. "Anziehung" dadurch zustande kommt, dass an der Seite, wo das Auge schwächer belichtet wird, eine tonische Schwäche eintritt, und die Bahn des Tieres infolge Überwiegen der Muskelkraft an der Gegenseite, nach der dunkleren Seite abgelenkt wird. Nun hat aber Buddenbrock weiterhin zeigen

können, dass dieser Lichttonus von den phototaktischen Reaktionen grundver-
schieden ist, die letzteren sind dem Lichttonus sozusagen superponiert. Dies wurde
an der Drehscheibe nachgewiesen, indem die Tiere hier Kompensationsbewegungen
gegen die Drehungsrichtung ausführen, was ebenso wie bei Insekten optisch
bedingt ist (Ausfall nach Exstirpation der Augen). Diese Kompensationsbewe-
gungen werden nun auch gegen die Wirkung des Tonusverlustes, der durch einsei-
tige Blendung entsteht, ausgeführt. Wenn z.B. das rechte Auge geblendet ist, ruft
die Verminderung des Tonus an derselben Seite eine Kreisbewegung im Sinne des
Uhrzeigers, also nach rechts hervor. Wenn ein solches Tier auf der Drehscheibe in
demselben Sinne gedreht wird, so muss es Kompensationsbewegungen der Richtung
des Uhrzeigers entgegen, also nach links ausführen. Und dies tut es tatsächlich
trotz des höheren Tonus der linken Körperseite. Durch weitere Analyse der
Lichtreaktionen (Phototaxis, und sog. "Lichtkompassbewegungen") wurden diese
Ergebnisse noch bestätigt. Buddenbrock bemerkt, dass eine ähnliche Überlagerung
phototaktischer Reaktionen über echte Lichttonuserscheinungen sich auch bei den
Versuchen von Holmes (1905), Carpenter (1905) und Rádl (1903) gezeigt hat, aber
von den erwähnten Autoren gar nicht gedeutet wurde, während Kafka (1914) in
seiner "Tierpsychologie" die Überwindung des Lichttonus von anderen Reaktionen
mit Einflüsse assoziativer Zentren erklärt. Diese letztere nicht-physiologische
Deutung kann jedoch nach den einfacheren physiologischen Erklärungen von
Buddenbrock nicht in Betracht kommen.

Wenn diese Ergebnisse schon die Bedeutung der erwähnten früheren Unter-
suchungen über Lichttonus einigermassen einschränken, so trifft dies noch mehr
für die Ausführungen von Garrey zu, die sub titulo Lichttonus noch viel mehr nicht
dazu gehörige Erscheinungen behandeln, als die Arbeiten, mit welchen Budden-
brock polemisiert. Offenbar waren aber Buddenbrock damals die Auseinander-
setzungen von Garrey noch nicht bekannt. Bald wurden aber auch diese selbst in
Angriff genommen, und zwar von Mast (1924), der nachgewiesen hat, dass die
Kreisbewegungen bei *Proctacanthus* nach einseitiger Blendung nicht durch ein-
seitigen Tonusverlust verursacht werden. Es gibt Fälle, wo die Tiere nach der
einen Seite überhängen und nach der anderen kreiseln. So verursacht z.B. Blen-
dung der Unterseite des linken, und der Oberseite des rechten Auges Neigung nach
der rechten und Laufen nach der linken Seite. Ferner wurde gezeigt, dass in
gewissen Fällen (Belichtung schräg von hinten) die Kreisbewegungen so ausgeführt
werden, dass die Beine an der belichteten Seite rückwärts, an der Gegenseite vor-
wärts schreiten. Das kann nicht auf eine quantitative Tonusdifferenz zurück-
geführt werden, sondern es sind qualitative Unterschiede zwischen den Bewegungen
der Beine an den zwei Körperseiten vorhanden. Die phototaktischen Bewegungen
sind überhaupt von Muskelkraft unabhängig, da sie auch nach Amputierung einiger
Beine wie normal ausgeführt werden. Bei seinen Versuchen mit partiell geblendeten
Raubfliegen hat Mast noch eine weitgehende Differenz in der tonisierenden Wir-
kung verschiedener Augenteile nachgewiesen, die mit den von Garrey festgestellten
nicht vollkommen übereinstimmen. Auch dadurch waren Unterschiede erzielt, dass
einmal auf weisser, dann auf schwarzer Grundlage experimentiert wurde, wodurch

die Stellungen der Tiere stark beeinflusst waren. Es wurde auch die von Garrey beobachtete Herabsetzung des Muskeltonus nach beidseitiger Blendung in Abrede gestellt: die Tiere sollen auch dann noch normale Körperhaltung einnehmen. Dies kann aber vielleicht durch Unterschiede der Versuchsbedingungen (z.B. verschiedene Adaptationszustände der Tiere) erklärt werden, da man an der Richtigkeit der Garreyschen Ergebnisse, die auf objektiven Beweisen (Kriechspurdifferenzen) beruhen, kaum zweifeln kann. Ebenso kann vielleicht der Widerspruch zwischen den Beobachtungen von Axenfeld (1899) und Garrey (1918) gedeutet werden, da die von Mast beobachtete Tatsache, dass der Helligkeitsgrad der Unterlage die Körperstellung der Fliegen stark beeinflusst, die Vermutung nahe legt, dass es sich dort um ähnliche Unterschiede handelte. Weitere Versuche von Crozier u. Federighi (1925 a, b), ferner neuestens von Welsh (1932 b) sind wiederum bemüht Lichtreaktionen der Tiere auf Phototonus zurückzuführen, dies scheint aber nicht berechtigt zu sein, da wiederum typische Reflexe als Tonus betrachtet werden, und so offenbar eine Mutatio elenchi vorliegt.

Zusammenfassend kann man sagen, dass viele Erscheinungen die früher als "Lichttonus" betrachtet und gedeutet wurden, nicht unter diesen Begriff gehören. Die Grundtatsache aber, dass es Lichttonus gibt, und bei den verschiedensten Tieren exakt nachzuweisen ist, bleibt eigentlich unberührt. Die Untersuchungen von Alverdes (1923)—fast gleichzeitig mit jenen von Mast erschienen—haben die tonisierende Wirkung der Augen bei *Cloëon*- und Libellenlarven wiederum in voller Klarheit gezeigt. Alle Tiere, die Alverdes geblendet hat, haben sich ähnlich verhalten, nämlich den Körper nach der sehenden Seite geneigt. Die schiefe Körperhaltung hat manchmal zu einer Neigung der Körperachse um 45° geführt. Die *Cloëon*-Larven waren besonders günstige Objekte, und die Herabsetzung des Muskeltonus an der geblendeten Seite konnte direkt beobachtet werden. Schwerkraft und Abschneiden einiger Beine beeinflussen diese schiefe Körperhaltung nicht. Das Schreiten geschieht in gerader Linie, wird also von der Tonusdifferenz nicht beeinflusst.

Neuerdings hat Alverdes (1926) auch bei Krebsen, und zwar bei den Garneelen *Leander* und *Processa* Lichttonuswirkungen nachgewiesen, und deren Interferenz mit den phototaktischen Bewegungen mit grosser Klarheit demonstriert. Dies ist umso wichtiger, da früher abgesehen von einigen Hinweisen von Holmes (1908), der bei *Gelasimus pugilator* nach starker Belichtung eine Zunahme des Muskeltonus beobachtet hat, wenig über den Lichttonus der Crustaceen bekannt war. Eine einseitige Blendung bei den erwähnten Garneelenarten setzt den Muskeltonus der Rumpfmuskulatur an der Gegenseite herab weil die Nervenbahnen sich offenbar überkreuzen. Dies scheint übrigens, wie wir gesehen haben, bei Arthropoden eine allgemeine Erscheinung zu sein, welche, wie Buddenbrock (1919 a) auch bemerkt, im Gegensatz zu den Verhältnissen bei Schnecken steht. Deshalb hängen die Tiere um ungefähr 45° nach der intakten Seite herüber, die Erscheinung verschwindet aber in 5 Minuten wieder. Eine Ablenkung der Schwimmbahn nach der Seite des verminderten Muskeltonus bleibt aber auch weiterhin bestehen. Diese Kreisbewegungen werden von phototaktischen Orientierungsreaktionen über-

wunden. Man kann allerdings die Lichttonuswirkung daran erkennen, dass ein-
seitig geblendete Tiere, gleichgültig ob sie. positiv oder negativ phototaktisch
abgestimmt sind, nach der geblendeten Seite abgelenkt werden. Wenn Phototaxis
und Tonusdifferenz gegeneinander wirken, so kommt es vor, dass Tiere durch
Lichttonuswirkung ihrem photischen Sinn entgegen, zur "nicht gewünschten"
Beleuchtung geführt werden, während im Falle des Zusammenwirkens von Licht-
tonus und phototaktischen Bewegungen so stark gekrümmte Schwimmbahnen
zustande kommen, wie sie durch Phototaxis allein nicht verursacht werden können
(Abb. 10).

Neuestens hat Friederichs (1931 *a*) bei Cicindelinen wieder Zwangsstellungen
nach einseitiger Blendung beobachtet, die unmittelbar nach der Blendung etwas
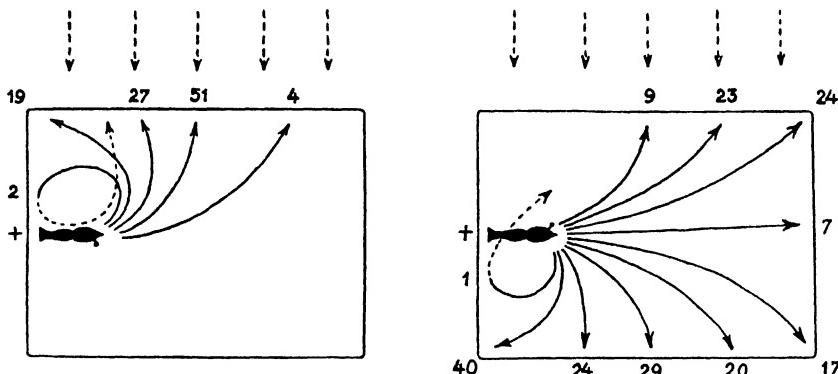

Abb. 10. Interferenz von Lichttonus und Phototaxis bei *Leander xiphias*, nach Alverdes. Links:
positiv phototaktisches Tier, einseitig geblendet, Lichteinfall von der geblendeten Seite (mit Pfeilen
angedeutet). Die Wirkung des Lichttonus verstärkt die Phototaxis, es kommen sogar übermässig
gekrümmte Laufbahnen zustande. Rechts: ähnliches Tier, Lichteinfall von der intakten Seite.
Lichttonus wirkt der Phototaxis entgegen, und das Tier wird oft trotz der positiven Phototaxis in der
Dunkelheit geführt. Die Zahlen neben den Laufkurven geben an, wie oft das Tier in den Versuchen
die bestimmte Route eingeschlagen hat.

anders ausfallen, als einige Tage später, im Wesen aber mit den früheren Feststellungen
übereinstimmen. Friederichs lässt jedoch die Frage offen, ob die Erscheinungen
mit der Deutung der Komplexaugen als tonischen Sinnesorganen in Zusammenhang
stehen.

(*b*) *Lichttonus und verwandte Erscheinungen bei Wirbeltieren.*

Über Lichttonuserscheinungen bei Wirbeltieren war bis zu den letzten Jahren
ziemlich wenig bekannt. Schon 1881 hat Högyes (1913) festgestellt, dass bei
hypnotisierten Personen die Belichtung der Augen gewisse tonische Reflexe auslöst.
Diese, nur ungarisch veröffentlichte Angabe blieb aber bis zu ihrer Übersetzung
ins deutsche 1913 vollkommen unbeachtet. Die Untersuchungen von Garrey (1905),
und hauptsächlich von Lyon (1904), haben so viel gezeigt, dass die Augen der
Fische bei den Reaktionen auf Strömungsreize eine Rolle spielen woraus man
schliessen kann, dass die Augen bei der Aufrechterhaltung des labilen Körper-

gleichgewichtes mitwirken und dies (wie z.B. Garrey, 1918, annimmt) durch toni-
sierende Wirkungen geschieht. Die Versuche von Loeb (1907) mit der amerikan-
ischen Eidechse *Phrynosoma blainvillei* ("horned toad") auf der Drehscheibe haben
ähnliche Ergebnisse geliefert. Es konnte gezeigt werden, dass die Kompensations-
bewegungen hier aus zwei Komponenten bestehen, von welchen die eine ebenso wie
die Kompensationsbewegungen der Insekten optisch bedingt ist, d.h. ruhende
Gegenstände werden während der Drehung fixiert. Bei Drehung von Tieren mit
offenen Augen fallen die Nachdrehungen nach Aufhören der Rotation viel geringer
aus, als bei geblendeten Tieren, und auch verschiedene andere Versuche weisen auf
das Vorhandensein solcher optischer Gleichgewichtsreaktionen hin. Pearcy u.
Koppányi (1925) haben neuerdings durch Translokation der Augen von Fischen
schiefe Körperhaltung der Tiere hervorgerufen, was auch auf die Gleichgewichts-
funktion der Augen hinweist.

Dass diese Funktion der Augen mit einer tonuserzeugenden Wirkung verbunden
ist war schon von vornherein höchst wahrscheinlich. Es wurde aber an den Augen-
muskeln der höheren Wirbeltiere auch experimentell gezeigt (Bartels, 1927).
Ferner soll das Rombergsches Phaenomen bei *Tabes dorsalis* einen durch das
Schliessen der Augen verursachten Tonusverlust darstellen (Schindler, zitiert nach
Metzger, 1931). Auch andere Erscheinungen aus der pathologischen und normalen
Physiologie des Menschen (Stein, zitiert nach Metzger, 1931) haben Einzelheiten
über den Lichttonus eruiert. Die verschiedenen Beobachtungen über die Kompen-
sation des Tonusverlustes nach Labyrinthexstirpation durch die Augen, spricht
auch für das Vorhandensein einer Lichttonusfunktion. Neuestens hat aber Metzger
(1931) zusammenfassend das Problem behandelt, und sehr eingehende Unter-
suchungen über den Lichttonus des Menschen und des Kaninchens ausgeführt.
Aus der Fülle der von ihm angeführten Tatsachen, von denen einige aber nur vom
ärztlichen Gesichtspunkt Bedeutung haben, sollen nur folgende Punkte hervor-
gehoben werden. Für die Versuche wurde eine sog. Tonusbrille konstruiert, in der
zwei Niedervoltlampen eingebaut waren, und eine diffuse Beleuchtung der Augen
erzielt werden konnte. Bei plötzlicher Beleuchtung des einen Auges, oder beim
Wechseln des Lichteinfalles war sowohl bei Kaninchen wie bei Menschen eine
Veränderung in der Körperhaltung zu beobachten. Wenn z.B. eine sitzende Ver-
suchsperson beide Arme nach vorne ausstreckt, so wird nach Einschaltung der einen
Lampe in der Tonusbrille die Hand an derselben Seite um 1–2 cm. höher gehoben,
an der Gegenseite um ebensoviel gesenkt. Auch Fallneigungen des ganzen Rumpfes
nach der belichteten Seite wurden beobachtet. Der Gang wird nach der belichteten
Seite abgelenkt, wozu bemerkt wird, dass diese Erscheinung beim Dressieren von
Cirkuspferden und Zugtieren, die ständig Kreisbewegungen nach der einen Seite
ausführen sollen, schon längst ausgenutzt wird, indem man solchen Tieren das nach
aussen sehende Auge verbindet. Versuche mit der Drehscheibe zeigen, dass der
Lichttonus bald additiv zu den Labyrintheffekten, bald antagonistisch diesen
entgegen wirkt, je nachdem, ob die Drehungen, bzw. Nachdrehungen der Tiere
(nach Anhalten der Scheibe) in, oder entgegen der Richtung des belichteten Auges
erfolgen. Es wurde ferner gezeigt, dass auch eine chromatische Differenz in Hin-

sicht auf diese Erscheinungen besteht, indem grünes Licht stärkere Tonuswirkung ausübt, als rotes von derselben Intensität. Bei Kaninchen wurde eine sehr auffallende alarmierende Wirkung des roten Lichtes nachgewiesen. Es wurden auch Versuche mit Belichtung verschiedener Retinateile ausgeführt, und gezeigt, dass die nasale Netzhauthälfte bei der Tonuserzeugung überwiegt, also ein ähnlicher Unterschied in dieser Hinsicht zwischen verschiedenen Augenregionen besteht, wie bei Insekten.

Obwohl nicht alle diese von Metzger als "Lichttonus" zusammengefassten Erscheinungen einwandfrei als Tonuswirkungen betrachtet werden können, zeigen sie ganz klar, dass die stimulierende Wirkung der Lichtsinnesorgane als eine ganz allgemeine Erscheinung im Tierreich zu betrachten ist.

B. *Photokinetische Erscheinungen und die Stirnaugen der Insekten
als spezifische photokinetische Stimulationsorgane.*

Nach dieser Übersicht müssen noch kurz einige spezielle photokinetische Wirkungen der Lichtsinnesorgane besprochen werden. Wir haben an betreffenden Stellen schon öfters darauf hingewiesen, dass diese Wirkungen von echten Lichttonuseffekten nicht vollkommen abgegrenzt werden können, und bei den als Lichttonus bezeichneten Erscheinungen eine ganze Reihe photokinetischer Wirkungen mitbehandelt wurden. Es sind aber noch verschiedene rein photokinetische Erscheinungen im Tierreich bekannt, von denen einige höchst wahrscheinlich auf Stimulationswirkungen der Augen zurückzuführen sind. Ein einwandfreier Nachweis hierfür fehlt jedoch, und wir werden uns deshalb nur auf wenige Tatsachen beschränken.

Photokinetisches Verhalten wurde u.a. bei ocellaten Medusen nachgewiesen, die im Licht viel beweglicher sind als im Dunklen (Romanes, 1885). Die statistische Registrierung dieser Erscheinung von Lehmann (1922) ist sehr überzeugend, man weiss nur nicht, wie weit die Photokinese von den Ocellen abhängig ist. Vielmehr kann man auf eine Wirkung des allgemeinen Lichtsinnes (photodermatischer Sinn) schliessen. Bei Crustaceen wurde ausgesprochene und graphisch registrierbare Photokinese von Davenport u. Cannon (1897), Yerkes (1900), und von W. F. Ewald (1910) an *Cypris, Daphnia* und *Leptodora* nachgewiesen. Intensität und Frequenz der Bewegungen (z.B. Antennenschläge) stehen mit der Lichtintensität in direkter Proportion. Die Ergebnisse wurden neuerdings von Siedentop (1930) an *Leptodora Kindtii* bestätigt, aber der Mechanismus der Wirkung blieb weiterhin sehr unklar. Ewald hat nur so viel nachweisen können, dass nach Zerstörung des Auges von *Daphnia* die Tiere nur kurze, rasche Antennenschläge ausführten, aber sich äusserst langsam dadurch fortbewegten. Dabei ist aber nicht sicher, ob diese üblen Folgen ausschliesslich durch die Augenzerstörung hervorgerufen wurden.

Neuerdings haben Moore u. Cole (1921) am Käfer *Popillia japonica*, ferner Cole (1921) an aufwärts kriechender *Drosophila*, und Welsh (1932a) an den Larven der "Muschelkrabbe" *Pinnotheres*, sehr ausgesprochene photokinetische Wirkungen nachgewiesen, die zahlenmässig mit der Lichtintensität zusammenhängen,

und anscheinend dem Weber-Fechnerschen Gesetz entsprechen. Es kann kaum ein Zweifel darüber bestehen, dass diese Wirkungen von den Augen ausgeübt werden, doch können auch photodermatische Erscheinungen an ihrem Zustandekommen beteiligt sein. Die Beobachtung von Knoll (1922), dass Schmetterlinge (*Macroglossa stellatarum*), die in einem belichteten Raum lebhaft herumfliegen, sofort zum Boden fallen wenn das Raum plötzlich verdunkelt wird, kann in ähnlichem Sinne gedeutet werden.

Bei Insekten wird manchmal auch das eigenartige Verhalten der Stabheuschrecke *Carausius* (*Dixippus*) als photokinetische Erscheinung betrachtet (Buddenbrock, 1924–28). Die Tiere verharren tagsüber in einer sog. Schutzstellung, während sie nachts auf Suche gehen. Dieses Verhalten welches als Katalepsie bezeichnet wird ist, wie Schleip (1911) nachgewiesen hat, vom Licht bedingt, da eine plötzliche Belichtung die Schutzstellung immer auslöst[1]. Über den Mechanismus der Lichteinwirkung auf die Katalepsie hat Stockard (1908) an *Aplopus mayeri* ("walking stick") der sich ähnlich wie *Carausius* verhält Untersuchungen angestellt, und gezeigt, dass hierbei auch ein allgemeiner Lichtsinn beteiligt ist. Die Ergebnisse, die von Schleip bestätigt wurden zeigen, dass die Blendung der Augen das Auftreten der Erscheinung nur verlangsamen, aber nicht vollkommen beseitigen kann. Hierbei machte Stockard die wichtige Beobachtung, dass wenn man nur die Komplexaugen blendet, die Verlangsamung der Lichtwirkung geringer ist, als wenn diese zusammen mit den Stirnocellen ausgeschaltet sind. Diese in der betreffenden Literatur völlig unbeachtet gebliebene Feststellung führt uns zum letzten, im Rahmen dieser Arbeit noch zu besprechenden Problem, nämlich zum Problem der Insektenocellen.

Diese in ihrer Bedeutung und Funktion so rätselhaften Organe der Insekten, die ebenso wie die Halteren der Dipteren von jedem Organ höherer Tiere so grundverschieden sind, sollen nach der neuesten Auffassung spezifische photokinetische Stimulationsorgane sein. Diese Auffassung wurde von Bozler (1926a) vertreten, der sie bei *Drosophila melanogaster* nachzuweisen suchte. Seine Ergebnisse haben gezeigt, dass Tiere, deren Facettenaugen sorgfältig geblendet waren, sich vollkommen wie blind verhielten, während solche denen nur die Ocellen geschwärzt waren, anscheinend gar keine Ausfallerscheinungen zeigten. Ein einziger Unterschied hat sich jedoch bei eingehenderen Untersuchungen wahrnehmen lassen, nämlich dass nach plötzlicher Belichtung die ocellengeblendeten Tiere sich nur sehr allmählich zu bewegen begannen, während die normalen sofort aktionsfähig waren. Auch das normalerweise ausgezeichnete phototaktische Orientierungsvermögen war nach Ocellenblendung—hauptsächlich bei niederen Lichtintensitäten—etwas geschwächt. Aus diesen Ergebnissen wurde gefolgert, dass die Erregungen der Ocellen keine eigenen Reflexe auslösen, sondern zur Stimulation anderer Reflexbögen, hauptsächlich der der phototaktischen Bewegungen dienen. Letztere Erscheinung, dass Stimuli spezielle Reflexe verstärken, ist analog jener, die bei einigen Schnecken vorzufinden ist, wo stereokinetische Stimuli zur Verstärkung der geotaktischen Bewegungen dienen (Baunacke, 1913). Dort wurde nämlich gezeigt, wie wir gesehen haben (S. 380), dass die statischen (geotaktischen)

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[1] Siehe Nachträge, 3, Seite 417.

Reflexe nur dann ausgeführt werden, wenn die Fußsohle vom Untergrund abgelöst wird, also die dort befindlichen taktilen Sinnesorgane stereokinetische Erregungen produzieren. Diese stereokinetischen Erregungen stimulieren also die Reflexbahnen der statischen Sinnesorgane, wodurch dann diese reflektorisch in Funktion gesetzt werden können.

Bozler versuchte bei der Begründung seiner Theorie die kurz vorher erzielten Ergebnisse von Homann (1924) über die optischen Verhältnisse der Ocellen auszuwerten. Nach diesen Befunden sind nämlich bei den meisten Insekten die Ocellen viel lichtstärker als die Fazettenaugen, und Bozler dachte nun, dass dies die Frage erklären kann, warum die photokinetische Wirkung der Ocellen ausgiebiger ist (z.B. in schwachem Lichte) als die der Komplexaugen. Trotzdem die Untersuchungen von Bozler durch die Befunde von Homann angeregt waren, hat er selbst die optische Analyse der Ocellen bei *Drosophila* nicht ausgeführt. Die spätere Nachprüfung (Wolsky, 1930) ergab dann, dass die Ocellen von *Drosophila* keineswegs lichtstärker, ja sogar bedeutend lichtschwächer sind, als die Komplexaugen. Dadurch musste die Theorie von Bozler dahin modifiziert werden, dass die Ursache der photokinetischen Bedeutung der Ocellen nicht in ihren optischen Verhältnissen, sondern in ihrer spezifischen Funktionsweise, also sozusagen in ihrem Wesen zu suchen ist.

Neuerdings hat zwar Müller (1931) festgestellt, dass bei Bienen (und vielleicht bei Ameisen) den Ocellen ein phototaktisches Orientierungsvermögen zukommt, dagegen die photokinetische Wirkung fehlt. Dies steht aber nur in scheinbarem Widerspruch zu der Auffassung von Bozler. Die Phototaxis der Ocellen besteht nämlich nur darin, dass sie die von den Komplexaugen ausgelösten Lichtreaktionen verstärken, dagegen die allgemeinen ungerichteten Bewegungsreaktionen unverändert lassen. Selbständig können aber die Ocellen auch bei Bienen keine phototaktischen Funktionen ausüben, sie sind also auch hier ausgesprochene Stimulationsorgane. Es scheint nur der Unterschied zu bestehen, dass die stimulierende Funktion der Ocellen bei Bienen, und eventuell bei Ameisen, vielmehr auf bestimmte Reflexbögen konzentriert ist, als bei *Drosophila*. Dort wurde aber übrigens, wie erwähnt, auch schon eine gewisse "Bevorzugung" derselben Reflexe durch die ocellaren Stimuli konstatiert. Diesen Unterschied kann man vielleicht teilweise damit erklären, dass die Ocellen der Bienen tatsächlich lichtstärker sind als ihre Fazettenaugen (Wolsky, 1931 a, b), teilweise damit, dass im hochentwickelten Bienenorganismus vielleicht auch die Ocellenfunktion schon höher differenziert ist, als bei anderen Insekten (vgl. hierüber die Auffassung von Bozler, zitiert von Wolsky, 1931 b).

Die neuesten Erörterungen von Friederichs (1931 b) geben beiden Auffassungen recht, so dass nach ihm die Ocellen einerseits phototaktischen, andererseits photokinetischen Reaktionen dienen sollen. Da aber zwischen den Feststellungen von Bozler, bzw. von Müller gewiss einige Widersprüche bestehen, scheint es viel wahrscheinlicher, dass es eigentlich zwei Arten von Ocellen gibt: solche, deren stimulatorische Wirkung mehr oder minder das ganze Bewegungssystem betrifft, und solche, deren Stimuli schon differenziert sind, und nur gewisse Reflexbögen

beeinflussen. Das Verhältniss, welches zwischen der Lichtstärke der Ocellen und der der Fazettenaugen eines Insektes besteht, gibt vielleicht einen Masstab dafür, ob man es mit "höheren" oder "niedrigeren" Ocellenfunktionen zu tun hat.

III. ZUSAMMENFASSUNG.

1. Im tierischen Organismus befinden sich Sinnesorgane, die als Stimulations-organe bezeichnet werden, und denen die Funktion zukommt, dem Zentralnerven-system solche Dauererregungen zu liefern, die zum normalen Betrieb des gesamten Nervenmuskelsystems, oder einzelner Reflexbahnen nötig sind. Die Bedeutung dieses Systems äussert sich einerseits in Erzeugung und Aufrechterhaltung eines gewissen Tonus der Muskulatur, andererseits in Aufrechterhaltung ihrer normalen Bewegungsfähigkeiten. Die erste Wirkung wird als Tonuserzeugung, letztere als kinetische Wirkung bezeichnet, beide können aber nicht streng auseinander gehalten werden.

2. Die meisten Sinnesorgane (vielleicht sogar alle) üben neben ihren spezi-fischen rezeptorischen Funktionen Stimulationswirkungen aus. Hauptsächlich kommen aber diese Funktionen gewissen niederen Sinnesorganen, ferner den ver-schiedenen statischen Sinnesorganen, und endlich den Lichtsinnesorganen zu. Unter den niederen Sinnesorganen gibt es auch solche, von denen auf Grund ihrer Wirkungen angenommen werden muss, dass sie ausschliesslich stimulatorische Funktionen haben.

3. Unter den sog. niederen Sinnesorganen üben hauptsächlich verschiedene mechanische Sinnesorgane eine bedeutende Stimulationswirkung aus. Dies wurde sowohl bei Wirbeltieren, als auch bei Wirbellosen nachgewiesen. Bei Wirbeltieren gelten z.B. die Erscheinungen des sog. Reflextonus als Stimulationswirkungen. Darunter versteht man jene Erscheinung, durch die verschiedene mechanische Sinnesorgane, in erster Linie die Organe des propriozeptiven Sinnes ("Muskel-sinn"), dann Sinnesorgane der Haut, der Eingeweide, u.s.w. dem Zentralnerven-system ständig Erregungen zuführen, wodurch ein ständiger Tonus der entsprechen-den Muskeln aufrecht erhalten wird. Wenn man die Erregungen irgendwie ausschal-tet (z.B. durch Narkotisieren der Nervenendigungen, Durchschneiden der sensiblen Nerven, u.s.w.), entsteht in den entsprechenden Muskeln ein Tonusverlust. Ähn-liche Erscheinungen wurden an verschiedenen Wirbellosen nachgewiesen. So wurde z.B. bei *Sipunculus* beobachtet (Uexküll, 1895), dass diese Tiere nach langem Verweilen in ihrer Röhre ihren Muskeltonus stark einbüssen, dass aber dieser nach mechanischen Reizungen bald wieder zurückkehrt. Die von Matula (1911) postu-lierte kinetische Stimulationswirkung mechanischer Sinnesorgane bei Insekten erscheint dagegen heute als unsicher.

4. Die sog. Stereokinese, d.h. eine allgemeine Mobilisierung des Muskel-systemes nach Ausbleiben gewisser mechanischer Reize, gehört aller Wahrschein-lichkeit nach, wenigstens teilweise zu den stimulatorischen Erscheinungen. Dies trifft hauptsächlich für Wirbellose zu, während stereokinetische Erscheinungen der

Wirbeltiere schon kompliziertere Funktionen sind, und kaum Stimulations-
wirkungen darstellen.

5. Von anderen niederen Sinnesorganen kommen vielleicht den chemischen
und den thermischen stimulatorische Funktionen zu.

6. Die Randkörper gewisser Medusen, die früher als spezifische Stimulations-
organe betrachtet wurden (z.B. Uexküll, 1901), üben nach der neueren Auffassung
keine derartige Funktionen aus (Bozler, 1926 b). Die Beeinflussung der rhythmischen
Bewegungen des Medusenkörpers, für die stimulatorische Wirkung dieser
Randkörper angenommen wurde, kommt in den Randorganen wahrscheinlich
automatisch zustande. Daneben besitzt der Medusenkörper auch eine spontane
Bewegungsfähigkeit, kann also in Hinsicht auf sein Funktionieren mit der Herz-
kammer der Wirbeltiere verglichen werden.

7. Die Halteren der Zweiflügler sind höchst wahrscheinlich spezifische Stimu-
lationsorgane (Buddenbrock, 1919 b). Ihre Ausschaltung verursacht nämlich keine
anderen Ausfallerscheinungen, als Tonusverlust und ein starkes Herabsetzen der
allgemeinen Muskelkraft (Flugunfähigkeit). Nach den Ausführungen von Budden-
brock (1920) betreffs der Untersuchungen Willes (1924), gilt dasselbe wahrschein-
lich auch für die Tibialorgane der Heuschrecke *Rhipipteryx chopardi*. Die früheren
Theorien über die Bedeutung und Funktion der Halteren (Steuerorganhypothese,
Gleichgewichtsorganhypothese, u.s.w.) reichen nicht aus, und können nach den
Untersuchungen von Buddenbrock (1919 b) als widergelegt gelten.

8. Die verschiedenen statischen Sinnesorgane haben eine dominierende stimu-
latorische Bedeutung im Leben des Organismus. Dies wurde zuerst vom Labyrinth
der Wirbeltiere nachgewiesen (Ewald, 1892 a). Die Entfernung desselben an beiden
Seiten verursacht einen starken Tonusverlust der gesamten Muskulatur, aber
hauptsächlich gewisser Muskelgruppen (Halsmuskulatur!). Auch verschiedene
kinetische Ausfallerscheinungen (z.B. Herabsetzung der Frequenz der Atembewe-
gungen) treten auf. Bei einseitiger Labyrinthexstirpation treten die Ausfaller-
scheinungen nur einseitig auf.

9. Später wurden dieselben Erscheinungen an den Statocysten verschiedener
Wirbelloser festgestellt. Die Ergebnisse sind ziemlich eindeutig. Nach Stato-
cystenentfernung wurde bei Würmern Tonusverlust (Buddenbrock, 1913), bei
Weichtieren durch Tonusverlust verursachte Bewegungsanomalien (Tschachotin,
1908) und eine messbare Tonusverminderung (Fröhlich, 1904 a), bei Crustaceen
ebenfalls zahlenmässig ausdrückbare Kraftabnahme und Tonusverminderung
(Bethe, 1897; Fröhlich, 1904 b) festgestellt. Den Gleichgewichtsorganen kommt also
ziemlich durchgehend nebenher eine Stimulationsfunktion zu.

10. Die Lichtsinnesorgane üben auch eine wichtige stimulatorische Wirkung
aus, deren Erscheinungen als "Lichttonus," bzw. "Photokinese" bezeichnet
werden. Diese Erscheinungen wurden zuerst bei Wirbellosen nachgewiesen, und
zwar bei Mollusken (Bohn, 1904 a, b) und Arthropoden (Rádl, 1903; Garrey, 1918).
Es zeigt sich nach ungleichmässiger Belichtung beider Augen, oder nach Entfernung
des einen Auges bei Mollusken ein Tonusverlust an der unbelichteten, bei Arthro-
poden an der belichteten Seite (Überkreuzung der Leitungsbahnen bei den Arthro-

poden). An der Aufrechterhaltung dieses Lichttonus sind verschiedene Augen-regionen in verschiedenem Grade beteiligt, wie dies an einigen Insekten gezeigt wurde (Holmes, 1905; Garrey, 1918; Mast, 1924).

11. Viele Erscheinungen, die als Lichttonus gedeutet wurden, hauptsächlich die verschiedenen Zwangsbewegungen (z.B. Kreisbewegungen nach einseitiger Blendung) können nicht auf solche zurückgeführt werden, und sind echte Reflexe des Nervenmuskelsystems. Dies wurde hauptsächlich dadurch gezeigt, dass man solche Bewegungen auch gegen Lichttonuseffekte erzielte (Buddenbrock, 1919a; Mast, 1924; Alverdes, 1926).

12. Später und hauptsächlich neuestens wurden auch bei Wirbeltieren, und sogar beim Menschen Stimulationswirkungen der Lichtsinnesorgane nachgewiesen (Metzger, 1931), die im wesentlichen mit den Erscheinungen bei Wirbellosen übereinstimmen.

13. Die photokinetischen Erscheinungen können von denen des echten Licht-tonus meistens nicht getrennt werden. In einigen Fällen (z.B. bei einigen Medusen nach Lehmann, 1922) sind jedoch spezifische photokinetische Wirkungen bekannt, über deren Natur aber zur Zeit noch kaum etwas ausgesagt werden kann.

14. In dieser Hinsicht sind die Ocellen der Insekten am besten untersucht, und sie stellen nach der heutigen Auffassung spezifische photokinetische Stimula-tionsorgane dar (Bozler, 1926a). Für diese Auffassung sprechen in erster Linie unmittelbare Versuche, ferner aber auch verschiedene optische Untersuchungen. Die in dieser Frage noch bestehenden Widersprüche können, wenigstens teilweise dadurch eliminiert werden, dass man annimt, dass die photokinetische Stimula-tionswirkung bei verschiedenen Insekten verschiedenartig ausgeübt wird, und an einer höheren Stufe (Bienen nach Müller, 1931) nur auf phototaktische Reflexe konzentriert ist.

IV. SUMMARY.

1. Stimulatory organs are sense organs, the function of which is to send continuous stimuli to the central nervous system. These stimuli are essential to the normal functioning of the nerve-muscle system or of certain reflex arcs. They are necessary for the production and maintenance of muscular tone and for the ability of muscles to contract normally. The former effect is known as the tonus-producing and the latter as the kinetic effect. The two effects cannot, however, be sharply distinguished from one another.

2. The majority of sense organs, perhaps all of them, have stimulatory functions in addition to their specific receptor actions. Such functions belong principally to certain "lower" sense organs, but also to the various kinds of static receptors and to light receptors. Among the lower sense organs there are some which, from their effects, must be assumed to possess nothing but stimulatory functions.

3. Among the so-called lower sense organs it is principally certain mechanical receptors which exercise an important stimulatory action. This has been demonstrated both for vertebrate and invertebrate animals. In vertebrates, for example, the so-called reflex tone is considered to be a stimulatory effect. By reflex tone is meant the maintenance of a permanent tonic contraction of muscles as a result of continuous afferent stimuli to the central nervous system from proprioceptive receptors and from receptors in the skin, viscera, etc. As soon as these stimuli are eliminated (*e.g.* by narcotising the nerve endings, sectioning the afferent nerves, etc.) a loss of tone supervenes in the corresponding muscles.

Similar phenomena have been described in a number of invertebrates. *Sipunculus*, for example, loses a considerable amount of its muscle tone when it remains for a long time in its tube, but the tone returns on mechanical stimulation (Uexküll, 1895). On the other hand, kinetic stimulatory effects of mechanical receptors, as postulated by Matula (1911) for the arthropods, do not seem to exist.

4. So-called stereokinesis, which is a general mobilisation of the muscular system as a result of the absence of certain mechanical stimuli, is probably, at least in part, a stimulatory phenomenon. This applies principally to invertebrate animals, for stereokinesis in vertebrates is more complicated and can hardly be of stimulatory nature.

5. Among the other lower sense organs, stimulatory functions are perhaps exerted by chemical and even more by thermal receptors.

6. The marginal bodies of certain medusae which were formerly regarded as specific stimulatory organs (Uexküll, 1901) have, according to recent conceptions (Bozler, 1926 b), no such function. The source of rhythmic movements in the medusae, for which a supposed stimulatory effect of the marginal bodies was made responsible, lies probably in autonomous centres in these marginal bodies. The body of a medusa possesses, moreover, a spontaneous rhythmic motility comparable with that of the ventricle of the vertebrate heart.

7. The halteres of Diptera are very probably specific stimulatory organs (Buddenbrock, 1919 b), the only result of their extirpation being loss of tone and a considerable diminution of muscular strength (ability to fly). According to Buddenbrock's explanation (1930) of the work of Wille (1924), this is also true of the tibial organs of the locust *Rhipipteryx*. Previous theories of the functions of halteres (steering, balancing, etc.) fail to explain the observed phenomena, and, as a result of Buddenbrock's investigations (1919 b), they can no longer be held.

8. The various static sense organs have a dominant stimulatory importance in the lives of animals. This was first demonstrated for the labyrinth of vertebrates (Ewald, 1892 a). The removal of both labyrinths results in a considerable loss of tone in the whole muscular system, and particularly in certain groups of muscles (neck muscles). Along with this loss of tone various kinetic deficiencies appear, e.g. diminution in the frequency of respiratory movements. Unilateral labyrinth extirpation causes only one-sided deficiencies.

9. Later on it was shown that similar phenomena are exhibited by the statocysts of various invertebrates. The extirpation of these organs has a similar effect in all groups of invertebrates which possess them. This operation causes loss of tone in worms (Buddenbrock, 1913), anomalies in movements (Tschachotin, 1908) and a measurable loss of tone (Fröhlich, 1904 a) in molluscs, and, finally, a similar measurable weakening of the muscles and loss of tone in crustaceans (Bethe, 1897; Fröhlich, 1904 b). Thus static organs have in general a stimulatory action in addition to their main function.

10. Optical sense organs have important stimulatory effects, known as phototonus and photokinesis. These phenomena were first demonstrated in invertebrate animals, namely in molluscs (Bohn, 1904 a, b) and in arthropods (Rádl, 1903; Garrey, 1918). When the two eyes of these animals are illuminated unequally (or one eye is removed) there arises a loss of tone in molluscs on the illuminated side, and in arthropods on the shaded side (due in the latter case to crossing over of nerve paths). Different regions of the eye have different degrees of importance in the maintenance of this phototonus, as has been proved for certain insects (Holmes, 1905; Garrey, 1918; Mast, 1924).

11. A number of phenomena which were formerly grouped under phototonus, particularly various forced movements (e.g. circus movements after blinding one eye), should not be placed in this category, since they are real reflexes of the nerve-muscle system. This view is supported chiefly by the fact that forced movements can be induced even when opposed by phototonus (Buddenbrock, 1919 a; Mast, 1924; Alverdes, 1926).

12. It has recently been proved that the eyes of vertebrate animals, including man, have a stimulatory function (Metzger, 1931) comparable with the phenomenon in invertebrates.

13. Photokinetic phenomena cannot generally be distinguished from true phototonus. In some cases, however (*e.g.* in certain medusae, Lehmann, 1922), specific photokinetic effects are known, but nothing definite can as yet be said of their nature.

14. In this connection the ocelli of insects have been best studied. According to the present view they are considered to be specific photokinetic stimulatory organs (Bozler, 1926a). This interpretation is supported both by experimental work and by optical investigations. Contradictions which still exist may be settled, at least partly, by assuming that the photokinetic stimulation manifests itself differently in different insects. Thus it may be assumed that in higher insects (bees, Müller, 1931) photokinetic stimuli influence phototactic reflexes alone.

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NACHTRÄGE.

1. Neuestens werden von G. Koller (*Berichte üb. d. wiss. Biologie*, **24**, 407) Einwände gegen dieser Arbeit erhoben, indem es nicht sichergestellt ist, ob die Stimulation tatsächlich durch Reizung der Tarsalglieder, und nicht etwa durch Reizung des Bauchmarks (welches von der ausstrahlenden Wärme der Unterlage nicht besonders isoliert war) zustande kommt.

2. Nach brieflicher Mitteilung des Herrn Dr G. Fränkel (Frankfurt a. M.) sind von ihm zur Zeit weitere Untersuchungen über die Halterenfrage im Gange, die obwohl noch nicht abgeschlossen, schon einige interessante Ergebnisse geliefert haben. Sie stehen in mancher Hinsicht im Widerspruch mit den Buddenbrockschen Anschauungen, teilweise werden aber diese bestätigt. Gegen Buddenbrock wurde festgestellt, dass die Halteren doch eine Rolle für die Erhaltung des Gleichgewichtes spielen. So kann z.B. der Verlust der Halteren durch Ankleben eines Stückchen Fadens am Abdomen kompensiert werden. Halterenlose Fliegen können mit diesen "Stabilisatoren" wieder koordiniert fliegen, während nach Abschneiden des Fadens sie plötzlich zu Boden fallen, weil sie das Gleichgewicht verlieren. Trotz dieser Ergebnisse kann die Stimulationstheorie der Halterenfunktion nicht aufgegeben werden, da normale Fliegen an einem Faden aufgehängt und frei in der Luft gehalten viel länger fliegen, als halterenlose. Ausserdem äussert sich die stimulierende Wirkung der Halteren auch darin, dass halterenlose Fliegen nur sehr schwer aufzuscheuchen sind.—Der Verf. dankt auch an dieser Stelle Herrn Dr Fränkel für die freundliche Mitteilung dieser Angaben.

3. Nach den neuesten Ausführungen von Steiniger (1933) ist die Katalepsie eine komplexe Erscheinung, in welcher neben photokinetischen Wirkungen auch andere Faktoren, wie Tonuserhöhung, heragesetzte Reflexerregbarkeit, u.s.w. eine Rolle spielen. Bei vielen Insekten sind ähnliche kataleptische Erscheinungen gar nicht vom Licht bedingt.

THE EVOLUTION OF THE CEPHALOPODA

By L. F. SPATH, D.Sc.

(With Thirteen Text-figures.)

CONTENTS.

I. INTRODUCTION.

THE development of the ammonites cannot be discussed independently of the evolution of their Palaeozoic forerunners, the goniatites, and the earlier types of these, again, are very intimately allied to the ancestral nautiloids. Although some-what diffident of casting so wide a net, I feel that the problem of the evolution of the Cephalopoda must be restated. In the past, what in my opinion are erroneous views of the development of the Ammonoidea have been popularised largely through the enthusiasm of workers who were attracted to the subject but had little practical experience of ammonites, and who too often were inclined to treat these fossils not as the remains of natural organisms but as material for speculation. This article, however, not only criticises the prevalent view that the ammonites, like the ancestral goniatites and *Clymenia*, arose from a straight nautiloid (*Bactrites*) and then hurried through the early stages of coiling before uncoiling again towards the end of their career; it also discusses various views put forward by workers on the primitive nautiloids. Yet with the purpose of making the article intelligible not only to the specialist but also to those who have only a slight acquaintance with the subject, I have attempted to illustrate in the text-figures at least the more important features of the different fossil Cephalopoda referred to in the following pages.

For the same reason I may be allowed to preface the article by a reference to what I have said elsewhere (1933 *a*, p. 703) concerning the speculative nature of geological science in general and the dating of our fossils in particular. The further back the geological historian goes, the more every unrecorded interval is liable to represent unknown centuries. I do not share the facile optimism of those who speak of the ease of observing successive mutations of animals, vertebrate or invertebrate. Few zoologists realise the magnitude of their jumps from say, "Pliocene" to "Miocene," from "Cretaceous" to "Jurassic." When, ten years ago, I wrote the first part of my *Monograph of the Ammonoidea of the Gault*, I was confident that the task would prove comparatively simple, and I believed myself to have the story of these fossils at my fingers' tips. Now the eleventh part of the monograph is in preparation and I am realising how many problems still remain to be solved. Yet it is doubtful whether there is any other Cretaceous formation in which clear and refined stratigraphy and abundance of good and unequivocal material afford equal opportunities for the description of so many successive ammonite faunas. The Jurassic is certainly far less thoroughly known, though some may be surprised to hear that great gaps still exist in our knowledge of the Jurassic faunas. The older the rocks, the more difficult it becomes to find uninterrupted successions. The number of Triassic ammonite horizons or Carboniferous goniatite zones may be imposing, but however excellent for stratigraphical purposes they are largely based on incompletely known species or else on isolated and disconnected forms. When we come down to the base of the Lower Devonian or the top of the Upper Silurian whence the first ammonoids have been recorded, our lack of exact knowledge is, indeed, appalling. It is necessary to impress upon the non-geological reader that this Lower Devonian—with two doubtful goniatite zones—was of incomparably longer duration than the whole of the Gault period—with its fourteen successive ammonite faunas—and that there is almost no material that is suitable or abundant enough for more detailed investigation of the less obvious characters. Even if sacrificed, the most favourably preserved, *i.e.* uncrushed, specimens, on account of the crystallinity of the matrix, would probably fail to reveal internal structure in thin sections.

But it is not the imperfect nature and doubtful dating of the material alone that suggest this warning. There is still much divergence of opinion on points which may appear settled to the general palaeontologist, judging by the text-books; such are the protoconch of "*Bactrites*," the nature of *Volborthella*, and others; and the interpretation of apparently simple features may vary as, for example, in the case of the annulation of certain forms of "*Orthoceras*." According to Ruedemann (1921, p. 320) this annulation "originated on the inside of the shells, by absorption, from the necessity of gaining more room to place the probably voluminous sexual products." To my mind, it is merely an economical way of strengthening the shell. While Sobolew's (1914) views on the clymenids, referred to below, have been considered by some to be too hypothetical to be even discussed, Schrammen's (1928) notion of a continual succession of shell-less cephalopods which repeatedly and independently acquired shells was not too fantastic to be published by a serious

scientific society. Many observers, bewildered by the seemingly endless variability of one small group, missed the essential uniformity of the ammonoid (or, indeed, cephalopod) stock as a whole.

But these examples may at least partly explain why the problem of the origin of the Ammonoidea and their relationship to the ancestral nautiloids is still debated. My own views outlined in 1923 (p. 65), are more or less diametrically opposed to Hyatt's (1867–1903); and it is attempted in the present article to show that some of the opinions expressed by Hyatt fifty years ago and still adopted by certain workers were based on mere conjecture. Thus it is still commonly asserted that (1) the recent *Nautilus* can be traced back in an evolutionary series through spiral gyrocones (Fig. 5 c) and curved cyrtocones (Fig. 5 b) to the straight *Orthoceras* (Fig. 5 a). This series is held (2) to be characterised by the absence of a calcareous protoconch. Then it is stated (3) that an *Orthoceras* in which the (supposititious) protoconch, *i.e.* initial chamber, had become calcified (instead of being shed) gave rise to a straight ammonoid shell ("*Bactrites*") with ventral siphuncle. Next (4) this is said to have produced the loosely coiled "*Mimoceras*" and the involute goniatites which in turn gave rise to the ammonites.

Assertion (1) was a fallacy even in Hyatt's time; he himself stated that there were no such serial relations in time. Assertion (2) was based on an assumption, discredited many years ago and now known to be quite untenable. With regard to (3), it can be demonstrated that every rich *Orthoceras* fauna (and sometimes a single species) has some forms with marginal siphuncle. (4) "*Bactrites*" (*recte Lobobactrites*) is a secondarily uncoiled goniatite because only series going from curved or coiled to straight can be demonstrated, never from straight to coiled.

When previously ridiculing the notion of a straight *Orthoceras* stage being "omitted" or "skipped" in *Nautilus* and the Mesozoic ammonites, I stated my conviction that the first goniatite, namely the Silurian *Agoniatites*, was merely a slightly modified *Nautilus* (*e.g. Barrandeoceras*), but my immediate concern was a disavowal of the usual palingenetic methods. Since then I have been prompted to discredit Hyatt's "laws" more vigorously, merely as a result of practical experience. Those who work on other groups may at least be interested to hear what constant handling of cephalopods for many years has—rightly or wrongly—suggested to me, as opposed to the theorising of amateurs at a distance. If it be objected that specialists like the late Prof. J. Perrin Smith (1932) came to different conclusions, I can only reply that in my opinion he constantly overlooked the fact that by heredity an ammonite was an ammonite, and that like other organisms it had to grow and therefore necessarily had to pass through more primitive stages (see Spath, 1933 b).

The principal conclusions arrived at in the present enquiry are summarised in the final section. Their briefness may invite categorical denials, but what is wanted is patient sifting of better evidence, if such be available. Much of the material for this article has been taken from my Catalogue of the Triassic Cephalopoda in the British Museum, now in preparation, and I must express my grateful acknowledgments to the Keeper of the Geology Department, Dr W. D. Lang, not only for allowing me to make use of this unpublished information, but also for enabling me

to draw so freely upon the unrivalled collections in his charge when preparing the hundreds of necessary sections.

II. DISTINCTIONS BETWEEN AMMONOIDEA AND NAUTILOIDEA.

Before tracing the ammonoids back to the nautiloids, it is necessary to reconsider the features of importance in the distinction of the two orders, for they cannot be culled from text-books. For instance, in such a popular work as Prof. A. Morley Davies's *Introduction to Palaeontology*, it is boldly asserted (p. 148) that the protoconch in the nautiloids (Fig. 5 e) is not calcareous and is nearly always shed. Yet it has been shown many years ago that the protoconchs of certain Ordovician, Silurian and Devonian *Orthoceras* were calcareous (Fig. 1). While I would not attach much importance to that unique Triassic *Nautilus barrandei* Hauer, figured by Jaekel (1902), in which the protoconch left the impression (on the succeeding whorl) of a spherical bladder, undoubtedly calcified, it clearly resulted from the observations of Blake, Barrande and Branco, of 50 years ago, that the existence of a conchiolinous protoconch in nautiloids, preceding the actual first chamber (Figs. 5 e, 6 a) was a mere assumption on the part of Hyatt. Schindewolf (1932) has recently simplified the problem by admitting that there was no essential difference in the form and development of the initial chamber in ammonoids and nautiloids. This difference, hitherto considered as of primary importance, is often dependent, in both orders, on the differences in coiling, and shape of the succeeding chambers or whorls, but there is naturally less variability in the tightly-coiled ammonites.

(1) *The initial chambers.*

The considerable differences in the dimensions as well as in the form of the protoconch of *Orthoceras*, demonstrated by Počta (1902, 1907), were discussed by Hörnes (1903), who also stressed the fact that they were free, *i.e.* not attached, as Jaekel assumed. Šulc (1932) has lately amplified Počta's work by observations on the calcareous protoconchs of *Orthoceras* from the Middle Devonian Hlubočep Limestone (Fig. 1 a–d); and Correns (1924) also figured protoconchs of his equally Meso-Devonian *O. clavatum* which differed from those of Clarke's (1899) Upper Devonian and Ruedemann's (1912) Ordovician forms. Schindewolf showed how the shape of such a calcareous protoconch may be determined by the shape of the succeeding chambers, and he maintained that in an elongated, longicone, form the initial chamber must necessarily contract at the upper end and become spherical or ovoid, whereas in the breviconic types the beginning is conical, the angle of growth remaining more or less constant. Unfortunately Schindewolf used for his illustration a median section published by Počta which Šulc now claims to be oblique; the latter author, moreover, objects that the brevicone forms may also be constricted, like the elongated shells. But those who compare Počta's original figures with the illustrations given by Šulc will admit that these protoconchs are extremely variable and that Schindewolf's main argument is not affected, although the modifications may not be mechanically "necessary."
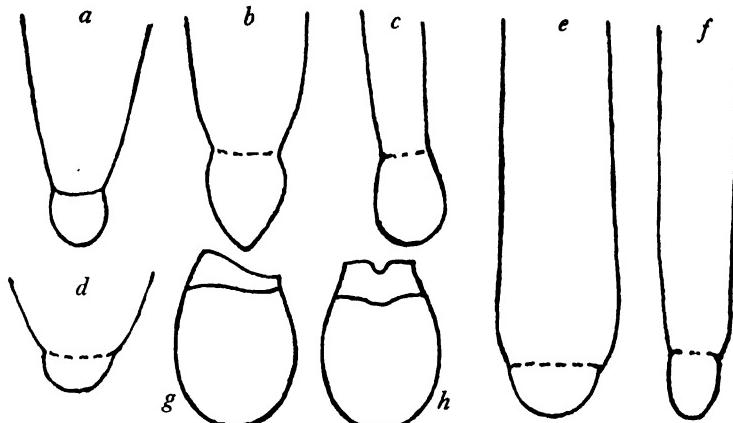
Fig. 1. Initial chambers of Devonian *Orthoceras* (*a–f*) and *Gyroceratites compressus* Beyrich sp. (*g, h*). After Šulc (*a–d*, ×16), Clarke (*e, f*, ×25 = "*Bactrites*"), and Branco (*g, h*, ×22). Note the difference in the first suture-line.
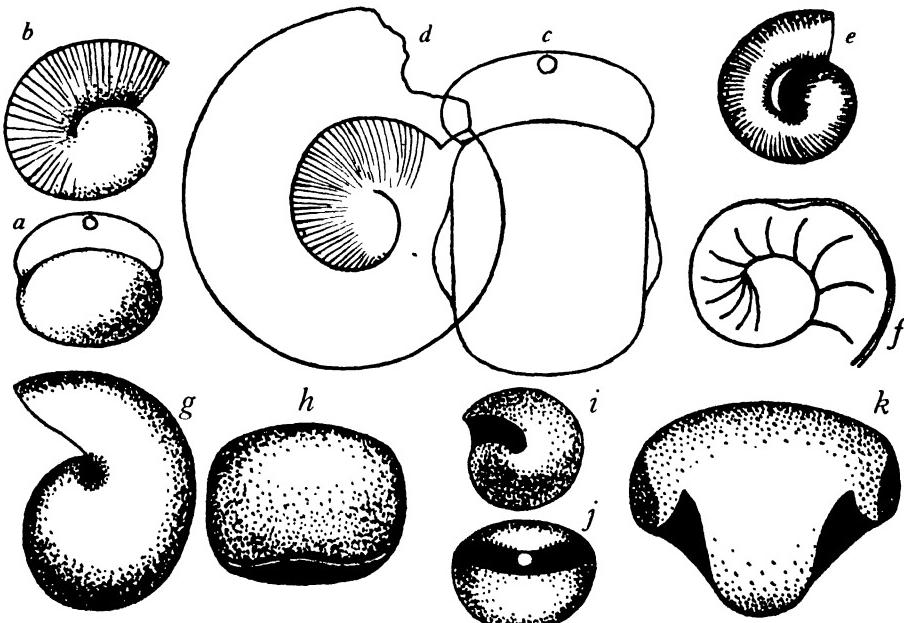
Fig. 2. Initial chambers of Ammonoidea. *a, b, Acanthoclymenia neapolitana* (Clarke): protoconch, with external siphuncle and striation on part of first whorl. *c, d, Anarcestes plebeiformis* (Hall): protoconch, with protruding ends, and striation (both after Clarke, ×20). *e, Anarcestes karpinskyi,* Holzapfel: showing umbilical perforation (after Holzapfel, ×6). *f, Anarcestes plebeius* (Barrande): section showing constriction (original, ×12). *g, h, Gephuroceras bisulcatum* (F. A. Roemer): side- and top-views of asellate protoconch. *i, j, Tropites subbullatus* (v. Hauer): side- and top-views of latisellate protoconch, showing external siphuncle. *k, Monophyllites simonyi* (v. Hauer): top-view of angusti-sellate protoconch. (*g–k*, after Branco, × about 45). *a, b, g, h,* Upper Devonian; *c–f,* Middle Devonian; *i–k,* Upper Trias.

The same applies to the ornamentation which was not observed by Šulc in his Bohemian *Orthoceras* material. It can easily be seen in nautiloids like a Silurian *Barrandeoceras*, a Carboniferous *Thrincoceras*, or a Triassic *Syringoceras*, that the surface of the initial chambers, in regard to the delicate, graceful, ornamentation, does not differ from the later portions; and it has been shown by Branco, Holzapfel, and Clarke that it is just in the oldest Ammonoidea (*Agoniatites* and *Anarcestes*), often with enormous protoconchs, that this young shell is also ornamented from the start (Fig. 2). In some specimens of *G. fecundus* from Barrande's collection, in the British Museum, the ornamentation also goes back as far as the protoconch, and I agree that the difference between Ammonoidea and Nautiloidea in this respect is only one of degree and without significance.

As the extreme types of initial chambers in *Orthoceras* are connected by various transitions, so in the coiled shells the shape of the later whorls often influenced the shape of the protoconch; and closely coiled nautiloid shells were in existence long before *Orthoceras* became a conspicuous element in the cephalopod fauna. Schinde-wolf considered the shallow, saucer-like, initial chamber of the recent *Nautilus* to represent a secondary condition, derived from a primitive, comparatively large and highly conical protoconch. I do not see the necessity for this assumption, for in the Ordovician already, forms with the coiling and general shape of the recent *Nautilus* are apt to show a similar initial chamber, reproducing that of the ancestral open, curved cones, like *Piloceras*, while in *Tarphyceras seeleyi* (Whitfield) (Fig. 4*a*), from the Tremadocian Beekmantown Beds, the many slender whorls and close coiling account for a beginning of the shell, scarcely distinct from that of Devonian goniatites, with a variable early stage (compare Fig. 2*e*). The differences in the protoconchs between Branco's "*asellati ammonitiformes*" (with a flattened spheroidal shape) and his "*asellati spiruliformes*" (with an egg-shaped, elongated protoconch) are not more striking than those between the first chambers of the Tremado-cian tarphiceratids and the contemporary nautiloids of the *Clymenia*-like Trocho-litidae; but while the umbilical perforation in various species of goniatites from the Devonian was figured by Sandberger as early as 1851, the inner whorls of similar nautiloids like *Tarphyceras seeleyi* are far less commonly known or easily obtained.

Between the extreme saucer- and egg-shaped protoconchs there are necessarily many conical, cup-, or thimble-shaped, transitional types; and nobody would insist on a direct or sudden transformation of the broadly conical initial chamber of, say, *Barrandeoceras bohemicum* (Barrande) into the spiral but large protoconch of an externally similar *Agoniatites* (see Figs. 10, 11). Apart from what is said below in refutation of the recapitulatorial significance of the unstable early stage, nautiloids (Barrandeoceratidae) with inner whorls much more like those of goniatites than the Upper Silurian *B. bohemicum* existed already before the Ordovician.

In this connection it may be mentioned that the initial stages are always more or less arcuate. Hyatt wrote in 1894 (p. 582) that when one considered the mode of growth of the young of any one of the straight or primitive arcuate forms of nauti-loids, the prominent fact was the bilateral symmetry of the cone and the asymmetry of the ventral and dorsal sides. He added that only subsequently, in the ontogeny of

the straight forms, in *Endoceras, Orthoceras*, the growth became more nearly equal. Now, to my mind, this merely indicates a different mode of existence in the young; but if the supposed "straight" beginning of one extreme individual of *Goniatites fecundus* was taken to point to a "*Bactrites*" ancestry, surely the arcuate apical cone of the straight forms should similarly have been considered to indicate a curved ancestor. The apical asymmetry, of course, cannot be applied to phylogeny any more than the variable depth of the protoconch, and Hyatt's "essential similarity" between the straight and the curved forms applies only to some, as shown below.

The protoconch in ammonoids also is variable. It may be as spherical in a Middle Devonian goniatite (Fig. 1g, h) as in an *Orthoceras* or in the Jurassic (Bajocian) *Spiroceras* (with dextral or sinistral coiling), and naturally it is more spindle-shaped in the majority of the tightly coiled ammonites, for the absence of brevicones in this order has already been insisted on by Schindewolf. In other words the later ammonites that have proved far too homogeneous a group to have been classified successfully up to the present, are also characterised by a protoconch that has remained almost unaltered, except on uncoiling. Variations in the size of the protoconch, however, still occurred in the Upper Cretaceous, as they occurred in the Devonian, without any obvious explanation, except perhaps the extremely loose coiling in *Anarcestes* as in *Gaudryceras*, with their large protoconchs. Otherwise the protoconch in Ammonoidea is dependent on the shape and coiling of the later whorls as much as in Nautiloidea and varies too much in goniatites, for example, to be of systematic importance. Even if not going so far as Schindewolf and insisting on the omission, henceforth, of the initial chamber from the diagnoses of the two orders Ammonoidea and Nautiloidea, it must be admitted that it has ceased to be an element of decisive importance.

(2) *The coiling.*

The gradual coiling of the early Cephalopoda, followed by a converse uncoiling in the Ammonoidea has come to be regarded in palaeontology as one of the most firmly established cycles of development. There is no need to go into the history of this cycle, but I may recall that at first (1867), Hyatt was rather vague about the *Orthoceras* ancestry and still considered all ammonites and goniatites to be derived from *Clymenia*, the latter being distinguished from the coiled nautiloid *Trocholites* (Fig. 4c) merely by its more discoidal shape. The "logical picture" of the cycle was elaborated only in Hyatt's paper of 1883 and especially in his well-known *Phylogeny of an Acquired Characteristic* (1894), and he there admitted that he had "deduced" the cycle of coiling or "morphic succession" from the individual development of the coiled shells. Hyatt, of course, was not unaware of the weak links in his chain of evidence, especially after the exact ages of the various forms became known, and his papers contain many contradictory statements and more or less doubtful admissions; but his followers made light of the difficulties. Overlooked was the fact that the first Devonian (or Uppermost Silurian) and the last Cretaceous ammonoids are typically coiled shells, as was Hyatt's own reservation that *Bactrites*, the supposed straight ancestor, may after all be an uncoiled goniatite.

As to nautiloids, it was asserted that in the Palaeozoic we reached in succession the straight orthoceratitic (Fig. 5 *a*), the curved cyrtoceratitic (Fig. 5 *b*), the loosely coiled gyroceratitic (Fig. 5 *c*), and the closely coiled nautiloid (Fig. 5 *f*) stages; yet again, Hyatt had pointed out that there were no such serial relations in time, that the typical *Cyrtoceras* showed a hereditary dorsal furrow and might have been derived from coiled ancestors, and so forth.

How all this was interpreted by the palaeontologist, however, may be seen from a short paper by W. D. Lang on the "Evolution of ammonites" (1919), in which he
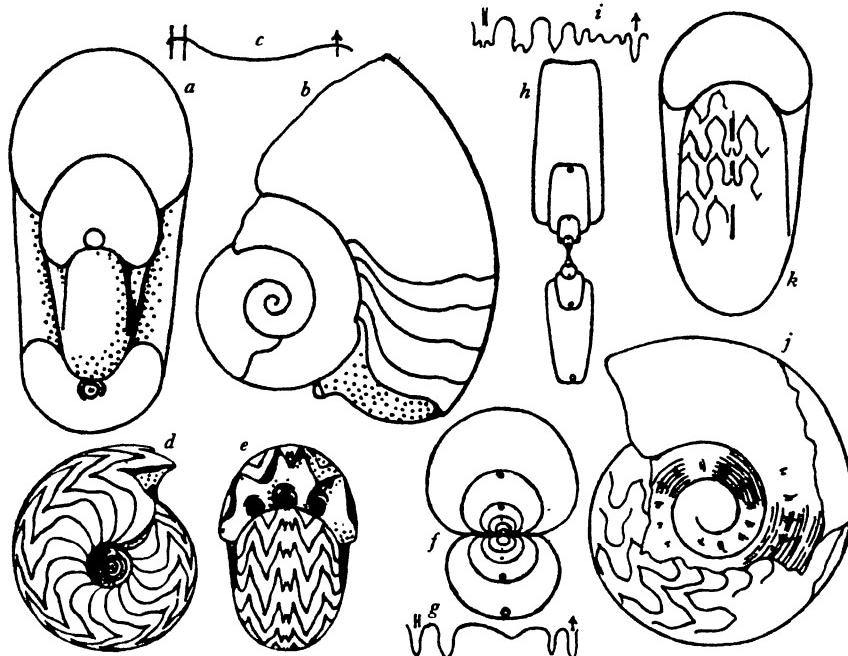

Fig. 3. *Clymenia* and goniatites. *a–c*, *Cyrtoclymenia angustiseptata* (Münster), with simple septal edge and large internal siphuncle. Upper Devonian, after Gümbel. *d–k*, four types of Carboniferous goniatites with external siphuncle (*Goniatites* s.s., *Aganides*, *Pronorites*, *Paragastrioceras*) showing different cross-sections and suture-lines. After Woodward, H. Schmidt, Foord and Crick. (All reduced to ⅔.)

traced the surviving *Nautilus* back into the Palaeozoic, and concluded: "Obviously, here is an evolution, starting with an orthocone and proceeding through cyrtocone and gyrocone to nautilicone." And, already, he is "prepared for this evolution not having occurred but once, but, on the contrary, being the normal sequence for every Nautiloid lineage." Then, following Hyatt again, he stated: "*Bactrites* is thus the primary ammonoid radical. We are prepared for a coiling of the ammonoid shell during further evolution similar to that occurring in nautiloids. So, along many lineages, there are loosely coiled forms, called mimocones, and tightly coiled ammoniticones." The latter statement must be due to a particularly serious misunderstanding, but it is repeated and rendered still more erroneous on pp. 56, 57:

"In all the main lineages of ammonoids, the shell, so far as the evidence goes, passed from a bactriticone through a mimocone, to an ammoniticone."

Hyatt, of course, never would have claimed anything like this sweeping generalisation, and A. E. Trueman (1922, p. 141) was careful to ascribe the omission of the hypothetical "straight" stage to "skipping," adding that in the Mesozoic ammonites "it could scarcely be said to be represented at all." Unfortunately, the enormous increase in the number of Palaeozoic nautiloid genera alone from a few to many hundreds makes it impossible for the non-specialist to get a clear view of what Hyatt really demonstrated, and what he is only assumed to claim, but the later expositions clearly show how misleading Hyatt's cephalopod "cycles" have become to the general palaeontologist.

But what must be insisted on is that Hyatt's cycle of coiling, put forward in the enthusiasm of the early evolutionary discoveries, was elaborated entirely on the basis of a few figures published by Barrande in 1865, but apparently not seen by Hyatt till after the publication of his first paper in 1867. The individual chiefly responsible for so much theorising was one of a number of young shells of *Goniatites fecundus* Barrande (1865, Pl. XI, fig. 4, magnified five diameters) in which the microscopic initial stage is unusually straight. Let it be noticed at once that there are other examples, undoubtedly of the same species, without trace of a straight stage, some being open spirals, some closed. The illustrations are not incorrect; the species is fairly common, although the individuals are crushed and the matrix is unsuitable for section cutting. It is also necessary to mention that the position and character of the siphuncle of the earliest goniatites are not known, and differences in coiling, in any case, were not considered by Barrande to affect the specific identity of the minute individuals, which, at any rate, are absolutely indistinguishable at larger diameters. Hyatt in copying Barrande's figures also included them under the one name *Agoniatites fecundus*, but why he did not apply his recapitulatorial reasoning to the concentrically coiled individual with spherical protoconch (Barrande's fig. 2), which did not fit into the cycle of coiling, was never explained. These two extreme forms as well as all the intermediate types came from the same bed, and there is no more evidence for assuming one to be a less primitive or later type than there is in the case of, for example, the Cretaceous *Scaphites circularis* (from bed X in the Upper Gault), discussed below. The innermost whorls of *Gyroceratites gracilis* (= "*Mimoceras*" *compressum*) are equally variable, which is important to note since a largely magnified and wrong drawing of the beginning of this goniatite has been so widely copied without mention of Branco's own correction.

Yet the configuration of the earliest whorls is still considered to be of importance in the distinction of members of the two orders Ammonoidea and Nautiloidea. Thus Schindewolf stated that the beginning of the shell in the Ammonoidea was always the most conservative element and was the latest to be affected by the subsequent modifications of the shell, and then not until a very late stage. But the very opposite might be claimed when uncoiling sets in. Of course, it is true that some heteromorph, *i.e.* uncoiled, derivatives of normal ammonites possess the spiral, transversely elliptical, protoconch of their ancestors and may retain one or even more complete

whorls before going off the regular spiral (Fig. 5 *h*, *i*). But this does not mean more than that an ammonite does not all at once change into another type of mollusc. In the Jurassic *Spiroceras*, in any case, the protoconch is spherical and detached, and the shell may coil dextrally or sinistrally, in a plane or in a helicoid spiral. It is only in the adult that perfect specific identity is observed. The earliest *Scaphites* known, namely the Cretaceous *Scaphites circularis* (J. de C. Sowerby, in Fitton) is almost an orthodox *Lytoceras* in most features, so that its pedigree is flawless, back to the
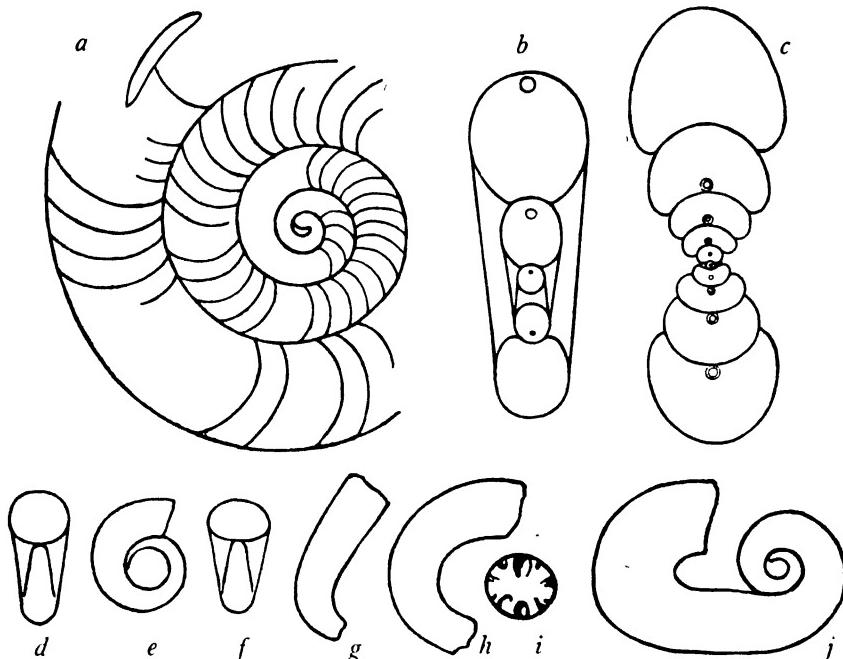

Fig. 4. Umbilical perforations in Nautiloidea and Ammonoidea. *a, Tarphyceras seeleyi* (Whitfield), with small umbilical perforation, $\times \frac{1}{4}$; *b, Tarphyceras multicameratum* Ruedemann, with subventral siphuncle, $\times \frac{2}{3}$; *c, Trocholitoceras walcotti* Hyatt, with dorsal siphuncle, $\times \frac{2}{3}$. (After Ruedemann, *a* and *c* Tremadocian, *b* Ordovician.) *d–j, Scaphites circularis* (J. de C. Sowerby). Upper Gault, *varicosum* zone, Folkestone. Outlines of involute and evolute varieties, *e* with large umbilical perforation. (*d* after Sowerby, in Fitton; others original; $\times \frac{1}{4}$.)

Lower Trias (*Palaeophyllites*). It is connected with the next higher *Scaphites* (*hugardianus* group) of the Uppermost Albian by the most perfect series of transitions (Fig. 4*j*), yet it shows an extremely unstable beginning, with the whorls coming into contact sometimes already at a few millimetres diameter or in other individuals scarcely touching at all, as in some associated hamitids. The protoconch, unfortunately, has not been found in this form, but there seems to me to be no reason why this unstable and most unconservative uncoiling of the young in such an ancient lytoceratid lineage should be ignored while a single individual of a comparatively new stock, *Agoniatites fecundus*, that happens to show a "straight"

beginning should have been selected by Hyatt from among its less elliptical and more closely coiled associates in support of a risky speculation. If there are no essential differences in the form and development of the initial chamber between the nautiloids and the ammonoids, surely the mode of coiling of the next whorl or more is of still less significance and equally adaptive.

Of course, the mode of coiling by itself in the young or adult has long since been given up as useless for indicating genetic affinity. Zittel, while recognising the weakness of Hyatt's classification, admitted already in 1884 that in discarding evolution or coiling as the chief characteristic, it was likely to give a truer picture of the genetic interrelations than previous classifications. But, as regards the Nautiloidea, Hyatt was again most contradictory. In 1894 (p. 368) he asserted that "all except the most primitive series, which were composed wholly of straight or arcuate forms, had some close-coiled species. These we could often trace directly with the greatest exactness both by their development and by the gradations of the adult forms, to corresponding species among the straight shells." Of course, it can easily be shown that the "most primitive series" were of Hyatt's own construction; even *Lituites* had then been recorded from the earliest (Ozarkian) strata. For his later series Hyatt relied upon such Carboniferous cyrtocones and gyrocones as were copied by him from illustrations chiefly by de Koninck. Nevertheless he not only failed to show why development must have been from straight to coiled, but also pointed out that the earliest nepionic substages did not have equal circular bands of growth even in true *Orthoceras*, and are never quite symmetrical on dorsum and venter. In other words the descriptive term "straight" is only applicable to the apical cone in a general way. It is the "straightness" of, say, half an egg-shell, transversely but obliquely bisected, which is the shape of Ray Lankester's (1883, p. 664, Fig. 75) primitive siphonopod (Fig. 12*j*), or of Verrill's (1896, p. 99) molluscan "archetype"; also of primitive *Clarkoceras* and *Piloceras*, and of the apical conch of many coiled nautiloids.

Let us examine, however, whether development need be from a straight to a coiled stage. For example, the three Devonian genera *Ptyssoceras* (cyrtocone), *Ptenoceras* (gyrocone), and *Anomaloceras*[1] (nautilicone) were united by Hyatt in his family Hercoceratidae, and we may agree that they are closely related. There can be no doubt, however, that they represent merely a morphic succession, showing an increase of coiling, and not a filiation; for *Ptenoceras* has its own cyrtocones, and the straightened outer whorl of *Hercoceras* suggests that in the same stock *Ptyssoceras*-like cyrtocones were also produced. The last (*Anomaloceras*) is more globose but with umbilical perforation and it is close to *Halloceras* and *Kophinoceras*; and the forms of the latter, with large and rayed ventral siphuncle, lead directly to the true *Cyrtoceras* (= *Cranoceras* Hyatt). Hyatt himself admitted that *Cyrtoceras* "may be degenerate and have arisen from coiled forms," and that the next stage in the uncoiling is presented by *Jovellania*. Anyone who has examined specimens of *Jovel-*

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[1] *Anomaloceras* Hyatt, 1883, is not taken to be preoccupied (by *Anomalocera* Müller, 1837 [1838?], or *Anomalocera* Westwood, 1842) as Cossmann (*Revue Critique*, 1900, p. 43) held; and *Hyatticeras* Cossmann (non *Hyattoceras* Gemmellaro, 1888) is a synonym of *Anomaloceras*.

lania must have been struck by the resemblance of these shells, whose straightness is clearly secondary, to the (similarly Devonian) forms of *Cyrtoceras* and *Gyroceras*, themselves secondarily uncoiled and with an equally rayed actiniform siphuncle, and peculiarly close septation.
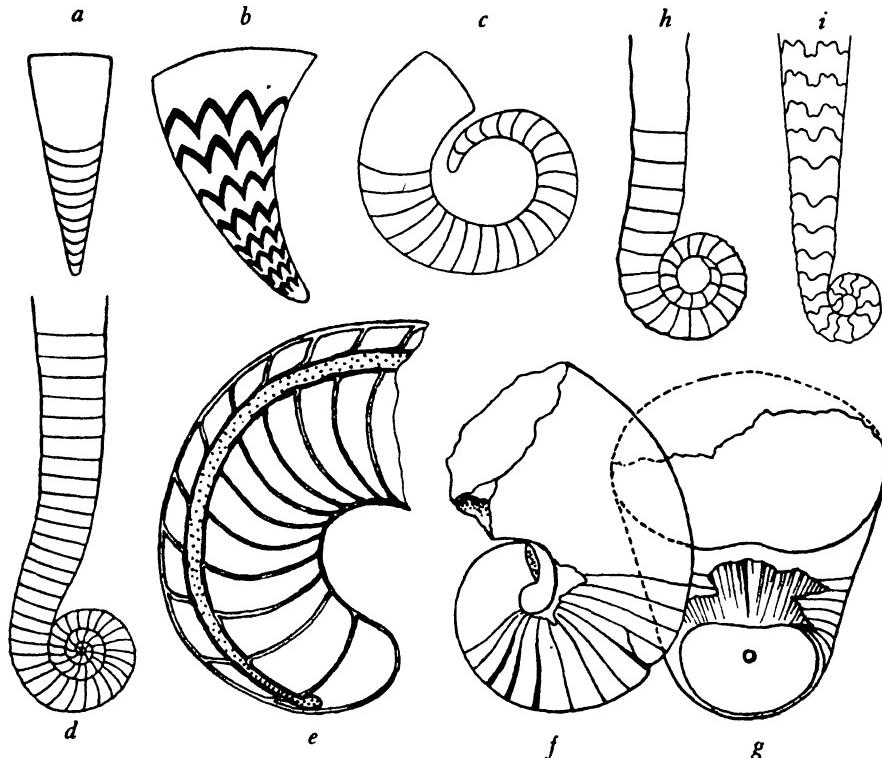

Fig. 5. Different types of coiling in Nautiloidea and Ammonoidea. *a*, Orthocone, *i.e.* straight shell. *b*, Cyrtocone, *i.e.* curved shell, with colour markings. *c*, Gyrocone, *i.e.* loosely coiled shell. *d*, *Lituites*, showing an early coiled and part of a later straight stage. *e*, Apex of a similar early nautiloid, *Estonioceras proteus* var. *demissa* Holm, showing siphuncle moving inward. *f*, *g*, inflated nauticone with impressed (concave) dorsal area, *Nephriticeras buccinum* (Hall). *h*, coiled beginning of a straight ammonoid, *Rhabdoceras*, in section. *i*, same stage in a *Baculites*. (*a, c, d, h, i* after Dollo, *b* after Ruedemann, 1921, *e* after Holm, 1898, *f, g* after Hall; *a–d, f, g* reduced, *e* enlarged ∧ about 4, *h* ×15; *i* ×12.)

Now, one of Hyatt's most important papers, the "Phylogeny of an acquired characteristic" (1894), is mainly concerned with what he calls "the impressed zone" or hereditary dorsal furrow. But *Cyrtoceras* has this impressed zone on the dorsum, so how can it be a "radicle of the Nautiloidea" as Hyatt stated (p. 368)? Indeed, when admitting that *Cyrtoceras* (= "*Cranoceras*") may be "degenerate," and its dorsal furrow and nephritic, *i.e.* kidney-shaped, outline (Fig. 5*f*, *g*) derived from a coiled ancestor, his only difficulty was to find "any pre-existing coiled form from which it could have been derived" (p. 598). In reality there are quite a number of

Lower and Middle Devonian genera that are closely allied and have a similar rayed, ventral to subventral, siphuncle, *e.g. Naedyceras, Nothoceras, Kophinoceras, Anomaloceras, Nephriticeras*, etc.; and on p. 520 (1894) Hyatt himself stated that *Cyrtoceras* (= "*Cranoceras*") appeared to be allied to the more closely coiled nautilian forms of *Naedyceras*. So that if Hyatt's "acquired characteristic" has demonstrated any genetic succession including both orthocones and nautiloid forms, it is this uncoiling Devonian series going from nautilian to straight. However, there are comparable series not only in the Devonian, but throughout the Palaeozoic, from the Ordovician Lituitidae to the Carboniferous Trigonoceratidae. If, then, we remember that the larval stage was a curved cone, and that, as in all tubular organisms, the shell grew by adding to the mouth, being thus free to continue in any direction, it must be admitted that it is unsafe to assume that the succession from orthocone to nautilicone is "the normal sequence for every nautiloid lineage." In fact, since only such series as go in the opposite direction can be established, we might almost go to the other extreme and say that secondary uncoiling accounts for the occurrence of those straight forms (at least in post-Ordovician times), that by peculiarities of shape, ornament, etc., were linked with nautilicones. In a word, like any other single character, it is inadvisable to take the coiling by itself as a working rule for all occasions; and looking at the evidence with an unbiassed eye, it seems to point to a cyrtocone as the primary ancestor of both the straight and the coiled forms among the Nautiloidea.

(3) *The siphuncle.*

The position of the siphuncle in nautiloids even in individuals, is very variable (Fig. 6). Barrande stated as early as 1855 (p. 160) that it was precisely on account of the invariable position of the siphuncle that he excluded *Clymenia* and *Goniatites* from the family Nautilidae, in which that element seemed to be essentially variable in form as well as in position. Branco (1879) later showed that this difference was confined to more advanced stages and that in ammonoids, at a minute size, the position of the siphuncle may also vary very considerably, but he was very careful not to attach too much significance to this variability. Zittel and Pompeckj also saw in this wandering siphuncle merely an indication of nautiloid ancestry. Now, however, Schindewolf (1931) put the position of the siphuncle in the forefront of all the characters of the young ammonoid shell, and attempted to show that most ammonites, including all the Jurassic and Cretaceous forms, must be derived from *Clymenia*, the goniatite stock having become extinct in the Triassic. He based this sweeping generalisation on what he considered the phylogenetic process of the gradual displacement of the originally dorsal siphuncle (in *Clymenia*) to an increasingly more ventral position, until finally the original condition was entirely skipped in ontogeny. This, of course, would indeed be an ideal example of acceleration, but it is not borne out by the facts.

To show the "extraordinary regularity" of the displacement of the siphuncle, Schindewolf jumped from the Lower Permian (*Agathiceras*) to the Upper Triassic (*Tropites, Trachyceras*, or *Sirenites*) and then settled the host of the later Mesozoic

ammonites with the sweeping but incorrect statement that the siphuncle had an intermediate position from the beginning. Apart from the fact that a classification based on a single character can never be either natural or even practicable, I am quite unable to see how Schindewolf could have drawn these far-reaching conclusions unless his material was very unrepresentative.

It is necessary to sift the evidence in some detail, in case other explanations than those here adopted could be advanced. Thus Schindewolf stated that, in the Jurassic and especially in the Cretaceous ammonites, the siphuncle did not originate in a dorsal position, but "in the majority of cases" began at once in a more or less central position to move very soon after to the external side. But I discovered the siphuncle to be in a definitely ventral position, not only at the beginning, *i.e.* where it leaves the caecum (for according to my observations this beginning is always more or less
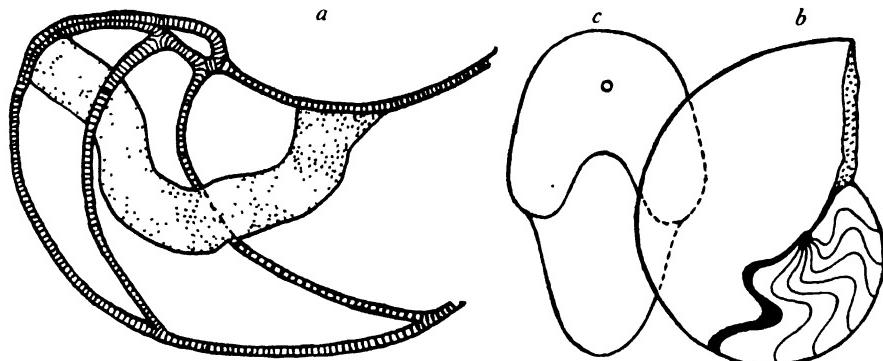

Fig 6. *a, Eutrephoceras dekayi* (Morton) An Upper Cretaceous nautilid, showing irregular siphuncle in first three chambers Greatly enlarged section, after Clarke (Compare beginning of siphuncle in the similarly unconstricted protoconch of Fig 5*e*) *b, Hercoglossa franconica* (Oppel), an Upper Jurassic nautilid with goniatitic suture-line. Reduced, after Zittel. *c*, sectional outline of a typical, occlusal nautilicone, with central siphuncle (reduced)

ventral, even in *Tropites*), but remaining in contact with the external side throughout, in genera as distinct as a Lower Liassic *Asteroceras* or a very late Cretaceous *Pseudophyllites*. Could this, perhaps, be ascribed to special "acceleration," instead of indicating derivation of these genera from *Goniatites* rather than *Clymenia*? Conversely, the siphuncle being dorsal for some time in an Upper Liassic *Harpoceras* or in a Middle Jurassic *Strigoceras* (Fig. 7*a*), when in the Lower Liassic *Psiloceras* it is close to the venter from the beginning, might possibly be explained by atavism, though it is difficult to see why in the late Cretaceous *Schloenbachia oregonensis* the siphuncle should not be ventral even at the diameter of 3·33 mm. (with three whorls), when in the Triassic *Sirenites*, according to Schindewolf himself, it became external already after 2½ whorls.

I may say at once that according to my observations the position of the siphuncle in the same species does not seem to vary much; for example, in *Promicroceras planicosta* from the Lower Lias the siphuncle may become external between the twentieth and twenty-fifth septum, as in the earlier *Microderoceras birchi*, but more

often it does not do so until about the fortieth septum. On the other hand, in the Yorkshire *Psiloceras erugatum* (Phillips) it is almost exactly the same as in the Alpine *P. calliphyllum* (Neumayr), being always close to the venter though not becoming quite external until about the twenty-seventh septum.

Again it may be granted that *Psiloceras* already represents an accelerated type, since in the much later *Phylloceras heterophyllum* the siphuncle, central at first, does not become external until after the fiftieth septum. But a first difficulty arises when such stocks as *Schlotheimia* and the Ammonitidae are examined, both considered by Schindewolf (and by everybody else) as derived from Psiloceratidae. For in *Schlotheimia (Scamnoceras) angulata* the siphuncle does not become external until about the fifty-sixth septum, and in the still later *S. (Charmassiceras) posttaurina* (Wähner) the siphuncle remains central to the forty-fifth septum and is still well away from the venter at the sixtieth septum, the last visible in my section. On the other hand, in the arietid derivatives of *Psiloceras*, the siphuncle may be at first well away from the venter (*Arnioceras nigrum* and *Arnioceratoides krideon*) or it may be absolutely ventral throughout (*Asteroceras* and *Oxynoticeras*); it may move slightly away from the venter only between the third and eighth septum in one *Cymbites* or be absolutely external from the start in another, while a third *Cymbites* may show a subventral siphuncle still on the third whorl.

It seemed possible that a ventral groove or keel might, at least partly, account for this difference, but I could get no confirmatory evidence. For in *Craspedites subditus* (Trautschold) from the uppermost Jurassic, for example, the siphuncle moved more quickly to the venter than in its presumable derivative, the keeled *Garniericeras catenulatum* Trautschold sp. (fiftieth septum). In the carinate *Hauericeras gardeni* (Bailey) again, as in early desmoceratids like *Puzosia* and *Beudanticeras* with equally thick siphuncle, it is central at first, before it moves to the venter at or after the twenty-fourth septum, but in the unkeeled *Varunaites varuna* (Forbes) the thin and slender siphuncle is absolutely external from the start. The very massive siphuncle of *Pseudohaploceras (Deshayesites?) aburense* again may be external already at the eighteenth septum, whereas in the later *Acanthoplites (nolani* group) it does not move to the venter until the thirty-fifth septum. The vagaries of the young siphuncle in the Jurassic and Cretaceous forms thus cannot be connected with whorl shape or ornamentation, and in any case, two examples of young *Lissoceras (oolithicum* group) from the Inferior Oolite, indistinguishable externally, have siphuncles that become ventral early (eighteenth) or late (not yet external at the last, sixty-third septum) (Fig. 7 c).

But the Triassic forms are more important from the systematic point of view, for if (as we all hold) *Phylloceras* is the root form of all later ammonites, its Triassic forerunners should be nearer the supposed Clymenid root stock. Unfortunately, here again, we find the evidence most contradictory. Thus in *Diphyllites* and a Timor form of *Leiophyllites* (Fig. 7 d) the siphuncle is external at first, and while in the former genus it moves away from the venter only very slightly (and temporarily) to become external again after the twentieth septum, in the latter genus it is well away from the venter at the fourth and still more so at the fourteenth septum (1½

whorls) to become external once more after the forty-fifth. *Discophyllites*, *Palaeo-phyllites*, and *Mojsvarites*, again, have external siphuncles, if not always at least at an early stage. *Monophyllites sphaerophyllus* has a constantly external siphuncle, while in the contemporary Middle Triassic *M. (Leiophyllites) taramellii* the si-
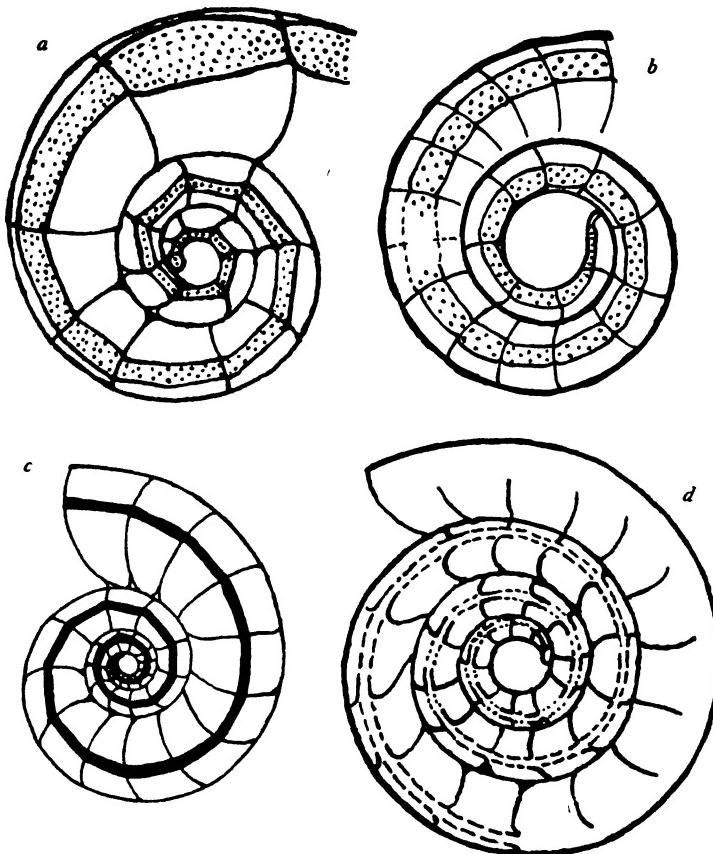
Fig. 7. Median sections of inner whorls of ammonites. *a*, *Strigoceras truellei* (d'Orbigny), Inferior Oolite, Dorset. Siphuncle at first internal, ×28. *b*, *Tropites torquillus*, Mojsisovics, Upper Trias, California, ×40. *c*, *Lissoceras* cf. *oolithicum* (d'Orbigny), Inferior Oolite, Dorset, ×20. *d*, *Leiophyl-lites* aff. *suessi* (Mojsisovics), Middle Trias, Timor. External siphuncle in first three chambers, ×28. (Originals.)

phuncle is still away from the venter at the sixty-sixth septum. In the Lower Triassic genera examined, it is still more difficult to account for the vagaries in the position of the siphuncle. For instance, while in some *Glyptophiceras* of the group of *G. minor*, from the Lower Trias of East Greenland, the siphuncle may be per-fectly external from the beginning, in others it moved temporarily away from the venter on the first or second whorl, while in the closely allied *Ophiceras demissum*,

from the same bed, the siphuncle may be definitely away from the venter, *i.e.* ventro-central to a diameter of 2·5 mm. In the more discoidal *Lytophiceras* the siphuncle is external or almost external at all stages, also in "*Meekoceras*" (= *Prionolobus*) and *Proptychites*.

I am not laying stress on the fact that in the Lower Triassic *Anasibirites multiformis* Welter, the siphuncle is almost external on the second whorl and that in *Columbites parisianus* Hyatt and Smith, and the Mesotriassic *Tropigastrites* ("*Celtites*") aff. *neumayri* (Mojsisovics) it is also external already at a very early stage, because the connection of both Sibiritidae and Celtitidae with the Tropitidae may be questioned. It is well known that in the Upper Triassic *Tropites* (Fig. 7 *b*), the siphuncle, internal at first, may not move out to the venter for a very long time (3¼ whorls), but I have found considerable variation even between *Tropites* and the compressed *Discotropites*.

Now *Tropites* has always been held to be derived from the Carboniferous Glyphioceratidae, though probably wrongly; but in these goniatites, in any case, the siphuncle may be either well away from the venter or else as close as Grandjean (1911) has drawn it for *Gastrioceras*. In the glyphioceratids from the Coal Measures the siphuncle, with the sheath continuous, often is of a rich golden brown colour in a clear matrix, and there is even possibility of confusion with the Liassic *Promicroceras planicosta*, if the labels were removed; but in the Lower Triassic Ophiceratidae and the Devonian *Timanites* the preservation may be equally good. Unstable siphuncles had long been figured in *Glyphioceras*, and were known to occur in *Dimorphoceras*, *Reticuloceras*, *Homoceras*, and other Carboniferous genera.

I suggested in 1919 that Clymenidae were derived from a goniatite ancestor with a siphuncle the position of which may have been unstable as in the early whorls of most latisellate ammonoids. I have no reason for distrusting Branco's researches on *Clymenia*, but in any case, Clarke had shown in 1899, that the earliest known *Clymenia* also had a ventral siphuncle at the start (Fig. 2 *a*) which moved to the dorsum only during the first whorl. Nevertheless Schindewolf declared categorically that there was never any shifting of the siphuncle in the goniatites and that "types with the unstable position of the siphuncle, postulated by L. F. Spath, without doubt did not exist."

Unfortunately most Devonian goniatites are preserved in crystalline calcite, if not pyritised, and do not show the siphonal sheath as do those from the Coal Measures and other beds mentioned above. Those *Anarcestes* and *Agoniatites* from the Lower Mesodevonian of Hlubočep (Bohemia) that are easily sectioned, show an external siphuncle from the start, as do *Tornoceras*, *Cheiloceras*, *Manticoceras*, and *Timanites*; but the (anarcestid?) ancestors of *Acanthoclymenia*, with presumably unstable siphuncle, are as yet unknown, and the Lower Devonian *Agoniatites*, etc., are far too rare and too poorly preserved to allow of investigation of their internal features. From the recapitulatorial point of view alone, I must doubt the existence of that hypothetical (Lower Carboniferous) "passage-form" between the Upper Devonian *Clymenia* and the Permian *Agathiceras*, stipulated by Schindewolf; but in the Middle Carboniferous Glyphioceratidae and Gastrioceratidae, in any case, the

siphuncle is known to be as unstable as in the Lower Triassic Ophiceratidae. Since these are at least as far removed in time from the Upper Triassic *Megaphyllites* (with permanently external siphuncle) as the latter is from the similar Lower Liassic *Asteroceras*, and since the impossibility of deriving some arietids from *Clymenia* and some from *Goniatites* must be apparent to everybody, it seems to me that Schindewolf's views cannot be upheld.

So far, we have considered only the instability of the siphuncle as regards movement in the plane of coiling, because this has been made the basis of a new classification of Ammonoidea. But movement in a direction away from this plane, leading to asymmetry, is common enough in ammonites and has also been interpreted as merely an abnormality that may attain a considerable degree of constancy but which was due to constitutional instability or to adaptation to a different mode of life. And although never considered of systematic importance, yet asymmetry of the siphuncle is even more likely to reflect fundamental differences than the unstable position in the early whorls, for without exception the latter is confined to a minute size, whereas asymmetry very often characterises the adult stages or, when found in the young, may disappear with the development of, for example, a keel. In an oxynote, *i.e.* very acute shell, like *Platylenticeras*, where asymmetry is constant, it may well be connected with bottom life; but in a round-ventered shell, like *Psiloceras*, the lateral displacement of the siphuncle may be due to individual instability, like the vertical displacement, or the great variability in septation, often noticed. Asymmetry is also know in goniatites, *e.g.* one of the Devonian *Tornoceras* I ground down had the siphonal lobe displaced to one side at diameters of between 10 to 20 mm.; and as the section shows the external siphuncle in corresponding positions to the innermost whorl, asymmetry must have begun in the larval stage. This is all the more surprising as even in perfectly median sections the siphuncle occasionally is lost for a distance, probably cases of helicoid siphuncular tubes, as noticed by Noetling (1906) in *Indoceras baluchistanense*.

I agree with those writers who postulate a change in mode of life after the ammonoid animal left the protoconch stage, *i.e.* when it ceased to drift as a planktonic larva. But on the earlier whorls the siphuncle is often of great width as compared with the later stages, decreasing occasionally from one-third to one-thirtieth of the width of the whorl; and as the massive and variable early siphuncle is combined with simple septal edges, as in nautiloids, it seems obvious to connect the two in our inquiry as to the function of the siphuncle and as to whether its variable position can be taken to be more significant in the Ammonoidea than in the Nautiloidea. Since I discussed this question in 1920, M. Schmidt (1925) has suggested that the pressure of gas in the chambers of *Nautilus* (and therefore probably the extinct ammonites) might have been regulated slowly but not quickly with the aid of the siphuncle and its blood-vessels, but to my mind the specific gravity of the shell and its inhabitant was so near to that of the surrounding sea water that it only required the slightest adjustment to rise or sink (in co-ordination, of course, with swimming movement in non-vertical directions). In the case of nautiloids, with large and complex siphuncle, it has even been maintained (Troedsson, 1926, p. 22)

that it helped to increase the gas-secreting surface, and thus served the same purpose as the folded mantle-edge of the ammonite animal.

There is no need to go in detail into the many siphuncular structures found in nautiloids, but it is well known that in the (polyphyletic) actinosiphonates, organic deposits in the form of "obstruction rings" were formed in the siphuncle, while the inconvenience of too great a buoyancy was counteracted in the endoceratids by loading the siphuncle with funnels or tabulae. The endoceratids in which this loaded siphuncle was marginal are often found facing downwards, as might be expected in presumably horizontally disposed swimmers; but in *Protocycloceras* the comparatively enormous siphuncle was more central and in the Lower and Middle Ordovician there are transitions not only to more typical orthoceratid forms such as *Bactroceras*, with a slender siphuncle in a marginal position, but also to the (epistomatous, *i.e.* upright) forms with central beaded siphuncles. In *Orthocerates* and even coiled forms ("*Discoceras*") again, too great buoyancy was averted by casting off the superfluous air chambers, altogether or in part, and sealing up the broken end (Barrande, Frech).

Beaded siphuncles were also produced over and over again. For example, the gigantic Carboniferous forms are certainly not descendants of either the Ordovician stock, or of the numerous Silurian adaptations known, with constricted mouth borders and similarly differentiated, central or marginal siphuncles. Again, in a form like the Silurian (Clinton) *Orthoceras erraticum* Foerste (1893, p. 236) there was not only constant variability in the position of the siphuncle in the same individual, but the earlier annulate structure became cylindrical in the later-formed part of the shell. In the ascoceratids the reverse change from orthochoanitic to cyrtochoanitic was probably due to a more abrupt change in the mode of life, but equally devoid of palingenetic significance. The slender siphuncle was purely adaptive and appeared more or less independently in the straight, swimming, crawling, or mud-burrowing forms as well as in the brevicones which lived in rock crevices, or in the forms with nautilian curvature.

As I have already stated (1920, p. 143), the assumption of gas secretion by the vascular siphuncle is not supported by the many fossil nautili in which it is separated from the camerae by thick mineral deposits. On the other hand, it is almost certain that the typically regular septation of Cephalopoda as a whole and the formation of chambers filled with gas (as distinct from tabulation in other tubular organisms) is dependent on a posteriorly attached siphuncle; for it is clearly of as much functional importance in the recent *Nautilus* as it was in a homoeomorphous Silurian shell, or in the latest Cretaceous ammonite (*Indoceras*, with constantly external siphuncle) as much as in a similar Devonian goniatite. And yet, it may be cut in *Nautilus*, without affecting its agility in the water (Willey). But my suggestion that the pull exerted by the siphuncle accounts for the progression of the end of the protoconch (gradually decreasing in size on the whole) from asellate to latisellate and angustisellate (Fig. 2) is not necessarily invalidated by the fact that in an Ordovician *Nautilus* (lituitid) the caecum may resemble that of an ammonoid (Fig. 5e), whereas in the recent *Nautilus* the siphuncle begins at the posterior end of the first chamber

and is scarcely dilated (Fig. 6a). For not only is the initial chamber flat and of similar size and the first septum thick and resistant, but there is no obvious change in the general shape and therefore mode of life as there is in the ammonoids, as between the protoconch and the succeeding chambers. But even if we differ as to the functions of the siphuncle, it seems clear that, as Kieslinger (1925, p. 102) has again shown, there is the greatest parallelism between the nautiloids and the ammonoids, reflecting not only their close relationship, but also similar functions of corresponding structures. In the immature ammonoids the siphuncle may have been more comparable to that of the nautiloids than in the adults, but in any case its vagaries in position at microscopic sizes were with good reason adjudged by the older systematists as of no more significance than the changes of position in the young *Eutrephoceras dekayi* (Fig. 6) or in *Cimomia imperialis*.
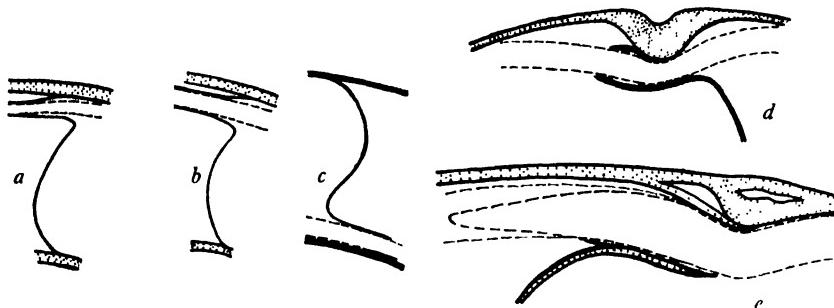

Fig. 8. Long, siphonal funnels of a, b, *Anarcestes plebeius* (Barrande), Mesodevonian, Bohemia, and c, *Clymenia* sp., Neodevonian, Shaldon, Devon. Constrictions and thickening of test in *Cheiloceras* sp. d, e, from the same locality, showing deflection of the siphuncle. (Greatly enlarged; all funnels are directed backward.) (Originals.)

Moreover, if the siphuncle is of such fundamental value in the Ammonoidea it is necessary to examine not only its position but its other and possibly far more important features. Thus there may be as considerable differences in the prosiphon and the caecum, already in the protoconch, as there are in the siphonal funnels and collars of the later septa or in the thickness in the adult. To my mind the development of the suture line in *Agathiceras* alone excludes the possibility of a derivation from *Clymenia*; but if Schindewolf (1931) considered the long funnels of *Agathiceras* as additional evidence in favour of a *Clymenia* ancestry it may be replied that *Agoniatites* and *Anarcestes* (Fig. 8) also have long backward funnels, only they are external; and even a nautiloid like *Aturia* could surpass *Cyrtoclymenia* in the length of its funnels (round its internal siphuncle) as late as the Eocene. Its resemblance to Palaeozoic nautiloids with similar siphuncle has never been taken to be more than merely an illustration of homoeomorphy. The funnels may be as long in some *Clymenia* as they are in *Agathiceras* or in one example of *Anarcestes latiseptatus* which I sectioned, but shorter or still longer in others, and anyone who examines a larger series of sections of clymenids will find that the length varies considerably. On the other hand, in *Cyrtoclymenia* the funnels (Fig. 9b) of the extraordinarily

wide siphuncle are holochoanoidal (as in an Ordovician *Endoceras*, Fig. 13 *a*, or in *Aturia*, Fig. 9 *a*) and they reach halfway beyond the previous septum; and these two nautiloid features alone would seem to suggest caution in referring this *Clymenia* to the Ammonoidea. The general palaeontologist, of course, who thinks of *Clymenia* merely as an ammonoid with dorsal siphuncle, is unaware of the enormous variety presented by the clymenids, not only in the less obvious features but also in the suture line, ornamentation and whorl shape. Hyatt (1883) already defined the whole sub-order of the *Clymeniae* as having characteristics so easily balanced that it would be difficult to decide whether it was nautiloid or ammonoid if it had not been for the protoconch and the young sutures figured by Branco. Now this author's illustrations of two examples of *Oxyclymenia* and Clarke's figures of the earlier *Acanthoclymenia*, also with an external siphuncle at first (Fig. 2 *a*), undoubtedly represent ammonoids and probably derivatives of some *Anarcestes* root stock. But in looking at some illustrations of *Cyrtoclymenia* in the literature, *e.g.* Wedekind's (1914, Pl. V, fig. 2) *C.* cf. *plicata* (Münster) or Gümbel's originals (Fig. 3 *a*), one wonders whether the essential uniformity of organisation in the two orders Ammonoidea and Nautiloidea could not mislead even the specialists. For I have before me four sections (Fig. 9 *b*) of Devonshire *Cyrtoclymenia*, including the keeled *Clymenia fasciata* (Phillips) (= *C. angustiseptata* Münster in Gümbel) with missing inner whorls that resemble, for example, the nautiloid *Trocholites* (Fig. 4 *c*) to such an extent that only the discovery of the centre in better specimens proved them to be clymenids. In other words *Clymenia fasciata* and its allies like *C. plurisepta* (Phillips) could well be nautiloids as far as the suture line, septation and wide siphuncle are concerned. But here, then, clearly all the features including the radial line, ornamentation, shape, and coiling fail to give even expert workers a clue to the real affinity of this group, and in view of the scarcity of material it is impossible to insist on the sectioning of every example of the Devonian *Cyrtoclymenia*, of the Carboniferous *Subclymenia* (a nautiloid with a ventral siphuncle) and other rare types, to make sure of their order. It is significant that Schindewolf (1924, p. 428) called *Cyrtoclymenia angustiseptata* with its slight differentiation the most frequently misunderstood and misidentified of all the clymenids. And if Sobolew (1914), with his large and well-preserved material, could seriously suggest that clymenids were only intrasiphonate mutations of normal goniatites (*Cyrtoclymenia* being paralleled with *Tornoceras*) the problem of the interrelations of the Palaeozoic ammonoids is not so simple as the text-books would have us believe.

The resemblance between *Cyrtoclymenia* and the nautiloids might be held to show that if any single character of the siphuncle be used for systematic purposes, it would have to be the width rather than the position. Only here again difficulties would be encountered at once in explaining the thinness of the external siphuncle in, for example, *Nautilus barrandei* (Hauer) or its great thickness in a young uppermost Cretaceous *Scaphites*.

As regards the thickness of the siphuncle in the later ammonites, it was at one time considered a distinction between "*Harpoceras*" and the oppelids, but, like the hollowness or solidity of the keel which may occasionally account for this difference,

it has not proved of value for systematic purposes. It is possible, on the other hand, that similarity in siphuncular thickness at small diameters in median sections may prove to be more characteristic, for while *Paraganides*, with its *Tropites* siphuncle, will probably be shown to have been incorrectly associated with similarly simplified offshoots of other stocks in a polyphyletic family, Nannitidae, *Varunaites* with its *Gaudryceras* suture line also has the slender external *Lytoceras* siphuncle. Unfortunately there seem to be too many transitional types to utilise the thickness of the siphuncle for more than very minor groupings.

(4) *Other characters.*

The protoconch, the siphuncle, and the coiling of the early whorls have been considered in detail because they have been taken to be of primary importance in the distinction of the two orders Nautiloidea and Ammonoidea; but differences in other characters, notably the configuration of the septa and their edges, are also generally quoted. It has further been stated that "the early coiled shells with a protoconch (*i.e.* Ammonoidea) are long, narrow, smooth, with septa usually far apart, and with a long deep body chamber; those without a protoconch (*i.e.* Nautiloidea) are short, broad, often with a longitudinal ornament, with septa relatively close together, and with a shallow body chamber" (Bather, 1911, p. 149). A wrong drawing of the earliest chambers of *Gyroceratites* ("*Mimoceras*") *compressus*, and a figure of the beginning of a recent *Nautilus* shell, given in illustration of this cryptic passage, only add to the reader's confusion. Again, in a more recent work (Schuchert, 1924, p. 529) we are told that the shells of ammonids are "nearly always more ornate, narrower or less deep and are often distinctly keeled along the 'centre' of the outer whorls" (than the pearly *Nautilus*) and that the siphuncle "in nautilids is near the centre of the septa but in the ammonids it is always placed near or in contact with the cone on its outer or ventral side." These ambiguous statements, of course, merely reflect the general uncertainty, ammonites not being the only cephalopods to form "a happy hunting ground for theorists."

In point of fact, whorl shape, except in the embryonic stage of some coiled forms, is of quite subordinate value, reflecting different modes of life, and as regards ornamentation it can be used for distinction only in a very general way; for there is almost as much variety among nautiloids as there was among the ammonites and similar interrelations among the various features. The presence of a keel, of tubercles, or of longitudinal ornamentation is of no more diagnostic value than an oxynote or grooved venter, though undoubtedly commoner in ammonoids than in nautiloids. Likewise the length of the body chamber, its modification in certain aberrant offshoots, and the form of the mouth border are too variable to be used for distinction, except in conjunction with all the other characters. The same applies to such less obvious features as the muscle impressions; and the discovery of aptychi *in situ* in ammonoids, although a real distinction so far as present evidence goes, is too rare to be of practical value.

The septum and its edge, however, generally afford a ready means of distinction between the two orders, but only in so far as the septal edge is never frilled in the

nautiloids. For wavy or angular suture lines occur in nautiloids as much as in ammonoids, and while the septal surface of a Devonian *Anarcestes* or *Agoniatites* (Fig. 10) is plainer than that of many a nautiloid (Fig. 6*b*), the complication of the suture line in, for example, a *Gonionautilus* exceeds that of many goniatites, clymenids, or even certain reduced ammonites, Triassic, Jurassic, or Cretaceous. I have discussed the value of the suture line on a previous occasion (1919, p. 177) though mainly in connection with the Ammonoidea, and I can only repeat that even if we place the suture line first, for systematic purposes, it can only be used in conjunction with the development of all the other characters of the shell. Applied to Nautiloidea, it thus seems to me that the suture line of an *Aturia* is of use in indicating its probable derivation from a hercoglossid; for the siphuncle may be internal even in a young *Cimomia* (which, of course, I do not take to indicate recapitulation of an
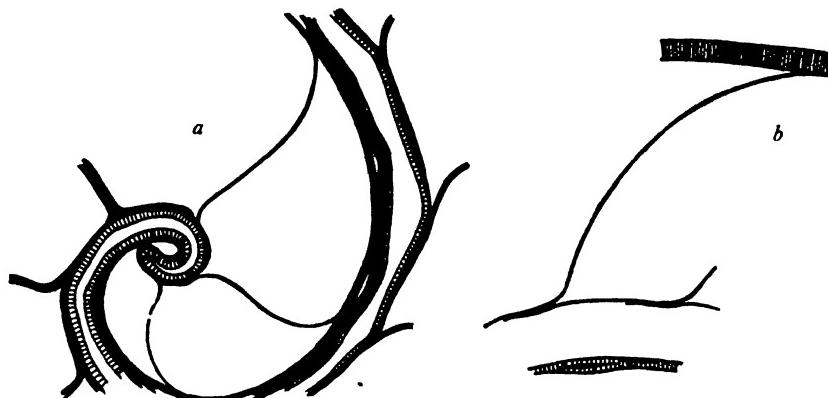
Fig. 9. *a*, section through inner whorls of *Aturia aturi* (Basterot), Miocene, Turin, showing internal siphuncle and long funnels (×20). *b*, *Cyrtoclymenia fasciata* (Phillips), Upper Devonian, Shaldon, Devon. Section through siphuncle, showing long funnels (×10). (Originals.)

ancestral state) and the long siphonal tubes of *Aturia* (Fig. 9*a*) can only have served to weight the shell. *Nautilus* itself persisted almost unchanged alongside these specialised offshoots, and since it has been considered a very primitive type on anatomical grounds also, we may accept not only a simple septal edge and a central siphuncle but also an unconstricted aperture and an open apical cone to represent original features.

So long as the function of the shell as a hydrostatic apparatus was not impaired, it mattered little in what way the changes in specific gravity during growth were adjusted. In each of the two examples of *Aturia aturi* which I have sectioned, the first three septa were very thin and the fourth of abnormal thickness, while even in the recent *Nautilus* an occasional septum (long before the last one) may be twice as thick as its predecessor, to be followed again by a thin one. In *Glyptophiceras minor* one individual may have only eight septa on the first 2½ whorls to thirty in another, and I have previously discussed similar irregularity in some adult ammonites. I may only add that in the young of *Arnioceras nigrum*, one of the species previously

quoted, the number of septa on the first three whorls may be fairly constant (twelve, seven, five or eleven, seven, five) but on the fourth it may change from four in one to nine in a second individual. In *Psiloceras erugatum* the number of septa on the second and third whorls may vary from seven and thirteen in one to five and six in another example, while a third has nine septa on each of the first three whorls. Clearly this is merely variability in individual growth.

But in a serpenticone shell with a vermiform animal (and a very long body chamber) the septa are sometimes very far apart in the adult, while in another, of similar build, they remain approximate; also in typical nautilicones with short and wide body chamber the septation may be either close or distant. And there is no reason why one species of *Orthoceras* should have very distant septa and another from the same bed be densiseptate, except that they were adapted to slightly different modes of life. Moreover, there are numerous individual differences in periodic thickenings, more or less hollowness of keel or tuberculation, etc., etc., affecting the shell as a hydrostatic unit, and the agility of its inhabitant in the water.

The formation of lobes in nautiloids is often connected with the siphuncle coming in contact with the outer wall; but in *Proclydonautilus goniatites* (Hauer) there are external and lateral lobes and yet the siphuncle is almost central. With Kieslinger (1925, p. 113) it may then be agreed that there is no statical necessity correlating the position of the siphuncle and the curve of the septal suture. Now one function of the folded septal edge probably was to give added strength to the shell while at the same time it reflects an increase in the posterior, gas-secreting, surface of the mantle. Since complication never occurred in straight or arcuate nautiloids it may be assumed that it indicates a tendency to a more ammonitic, *i.e.* less benthonic or at least less shallow water, mode of life. This does not seem to be borne out by the complex septal edge of some secondary straightened out ammonoids; for example, in *Diplomoceras cylindricum* the suture line remains extremely elaborate, but this hamitid form is derived from a stock (*Lytoceras*) in which the shell was as thin as paper and clear as glass, and there was no strong corrugation or tuberculation as in similar ancyloceratids. Whether these hooked forms were particularly good divers or altogether poor swimmers matters little; they were flourishing enough for long periods to warrant the assumption that they were well adapted to their particular environment. Moreover, as I have previously stated, the penetrating fibres of the folded mantle margin in all ammonoids, coiled or straight, afforded an additional means of attachment, not necessary in the (presumably) less agile nautiloids where the forward move of the shell muscles was gradual. So long as simplification of the saddles, *e.g.* in *Pseudoceratites*, was compensated by an increase in the number of lobes, it was of very slight significance; and reduction to a goniatitic type in ammonites was always accompanied by dwarfing and modification of the body chamber, *i.e.* a very distinct change in the mode of life.

Of still less importance therefore must have been the exact mode of formation of new lobes at the umbilical suture, or just inside or outside of the line of contact of successive whorls; for such development must have been different in involute and evolute varieties of one and the same species. Also, sometimes two lobes

appeared simultaneously at the umbilical suture, but not necessarily on opposite sides in the same individual; it matters little whether we call the inner or the outer U_1 or U_2, the symbol U being used only for an umbilical lobe in a very general sense. This peculiarity of the mode of formation of new elements in the suture line at the umbilicus has been made the basis of a new classification of ammonites, but, like any other single feature of the septation, it is of no systematic value.

Septation, by itself, is then of no more fundamental importance in ammonoids than in nautiloids; and a classification based solely on the characters of the septal edge is as likely to lead to unnatural groupings as one based on any one of the other characters here discussed.

Reference may finally be made here to an obscure character of the innermost whorls in Ammonoidea, namely, the constriction of the first whorl which, I agree with Grandjean (1911, p. 512), marks the end of the embryonic stage. But, according to this author, it is strongly marked in all ammonites and goniatites and generally occurs at between 270° and 290°, although it may vary from 260° in *Acanthoplites* to 375° in *Gastrioceras*. Grandjean therefore argued that the angle was greater in the Palaeozoic forms and diminished in the Jurassic and Cretaceous, but the great variability that has been noticed in all the other characters seems to apply also to this feature of the first constriction. In many ammonites I have not been able to see it at all, and in others it is so indistinct in median sections, that, if not specially looked for at the angle indicated, it could not be distinguished from the irregularities in the spiral that mark many young whorls. Again, in various Cretaceous ammonites I have found the angle to be as high as 360°, and in at least three genera (*Pseudohaploceras*, *Gaudryceras*, and *Varunaites*) there was a faint constriction at 340° followed by a deep one at 360°. The irregularities noticed in the Triassic and Palaeozoic forms also suggest that the distance between the first constriction and the protoconch would vary as much as the length of the body chamber in the adult.

III. THE AMMONOID ANCESTOR.

It is an established fact that the earliest goniatites only differed from most nautili in the closer coiling of their inner whorls; the position of the siphuncle, which became permanently established on the external side in goniatites, is of minor importance although it led to differentiation of the ventral portion of the suture line, or, at least, became associated henceforth with the region of greatest complexity of the septal edge. Zittel was thus perfectly justified in laying stress on the insignificance of the differences that separated the Ammonoidea from the Nautiloidea and it is apposite to recall that in the last edition of his *Grundzüge* (1924), undoubtedly the most widely used text-book of palaeontology, it is still stated that it was entirely uncertain from what form, or more probably what forms among the Nautiloidea, the Ammonoidea were to be derived. Frech (1902), also long after Hyatt had published his theory, regretted that the late appearance of *Bactrites* (according to him possibly a secondarily straight shell) did not allow of a decision of the problem as to whether the ammonoids were derived from straight or from coiled ancestors. The

only point that seemed to him to be in favour of the first view was that the Silurian nautili had already reached a high degree of differentiation and had nothing more in common with the simple Devonian goniatites. Even this point, at the present day, carries no weight, for the slight increase of coiling that would transform a nautiloid like *Tarphyceras* (Fig. 4) with ventral siphuncle into a true ammonoid shell is negligible, as are the correlated changes. Unfortunately, however, Silurian coiled nautili are as yet very incompletely known, and in associating the earliest *Agoniatites* with *Barrandeoceras*, I had in mind merely some true Silurian nautilian group.
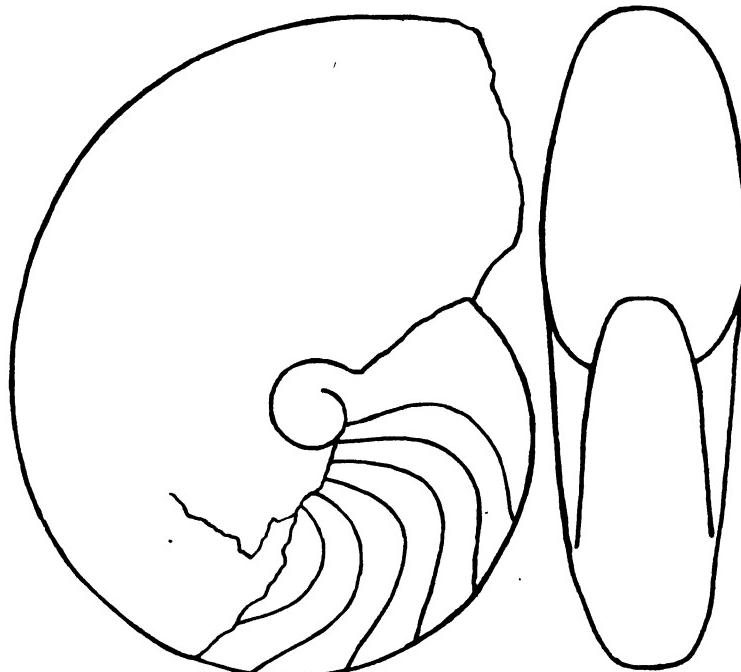

Fig. 10. *Agoniatites fidelis* (Barrande), an early (Lower Devonian) goniatite from Konieprus, Bohemia. Outline, with simple sutures (reduced ×⅔). (After Barrande, 1865, Pl. VIII, figs. 21–22.)

Some of these compressed, truncate, shells with broad lateral lobe differ very slightly in external characters from the similar Lower and Middle Devonian *Agoniatites* of, occasionally, very large dimensions (Fig. 10). There is no essential difference in the structure of the siphuncle, holochoanoidal (Foerste) or orthochoanoidal (Troedsson) in the original tarphyceratids (*i.e.* *Barrandeoceras* s.l.) with its septal neck pointing backwards. The differences in the course of the lines of growth (with a hyponomic sinus) are also unimportant. Among others, figures published by Barrande (1877, Pl. 476), Remelé (1890, Pl. III, fig. 3), and Mojsisovics (1873, Pl. XIII), will show how the radial line in nautiloids which have a tendency to biangular venters or latero-peripheral processes is modified exactly as in goniatites of externally similar form.

The genus *Barrandeoceras*, as founded by Hyatt in 1883 (p. 299) was comprehensive, but was later (1894) included in the family Tarphyceratidae, characterised by compressed shells with more or less ventral, tubular siphuncle. Foerste (1925, p. 7) suggested its inclusion, at least provisionally, in a separate family Barrandeoceratidae on account of the more central position of the siphuncle. But this character is not of generic value, in, for example, *Tarphyceras* itself, and sometimes not even specific ("*Orthoceras*" *koninckianum* d'Orbigny); and without sacrificing a number of valuable specimens of these nautiloids it is impossible to demonstrate
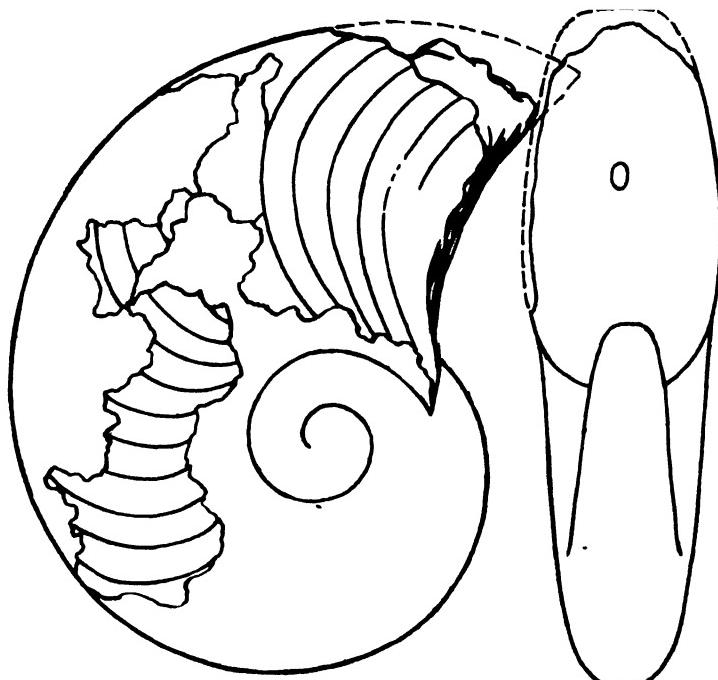
Fig. 11. *Barrandeoceras bohemicum* (Barrande). An Upper Silurian nautilid, from Bohemia. Outline, restored and reduced, ×¾. (After Barrande, 1865, Pl. XXXIV, figs. 1–2.)

afresh the vagaries of their siphuncles. In any case, the late Bohemian forms such as *Barrandeoceras bohemicum*, here figured (Fig. 11) and included by Hyatt in his genus, may perhaps be separated generically from the earlier costate type which according to Ruedemann (1906, p. 454, Pls. XXXII–XXXIII), occurs in the Chazy Formation (Lower-Middle Ordovician). The siphuncular structure of many of these forms, however, is not known and the transfer of "Barrandeoceratidae" and other types from one sub-order to another, reflect classificatory chaos rather than a workable application of Hyatt's elaborate if superficial scheme.

Hyatt himself traced the Silurian forms referred to *Barrandeoceras* (of which at least *B. bohemicum* has an annular lobe) back to their Ordovician (Chazian) forerunners, and they do not really differ more among themselves than do species of

later nautili. In any case, whatever the interpretation of *Barrandeoceras*, in their initial chambers, the Ordovician forerunners of this nautiloid genus are occasionally much more like *Agoniatites* than are the later, Silurian, forms; and it is well to remember that, after all, from the Lower Devonian onward, the nautiloids were on the decline. There is, for example, the Lower Ordovician genus *Eurystomites* Schröder (with ventral or subventral siphuncle) which has been described by its author as completely involute. The inner whorls of *Tarphyceras seeleyi* (Fig. 4 *a*), as has already been mentioned, show an agreement with those of early goniatites as close as that existing between the apical cones of the Ordovician contemporaries *Schroederoceras* and *Estonioceras* (two genera with very variable siphuncles) and the early chambers of the recent *Nautilus* (Fig. 5 *e*). In any case, neither the form of the protoconch, nor the closer coiling that caused it, can be used as an argument against associating the earliest goniatites with their nautilid contemporaries or forerunners.

Before examining how the later *Bactrites* and *Gyroceratites* (= "*Mimoceras*") came to be regarded as the ammonoid ancestors, I may add that there are many later offshoots of the Nautiloidea which developed either marginal siphuncles or goniatitic suture lines, but these never produced another ammonite radicle. It has been rightly claimed that the Upper Triassic period coincided with a second acme of nautiloids, after the first and more important acme in the Upper Silurian; and attention has already been directed to the striking parallelism in the development of the contemporary ammonoids and nautiloids, obviously indicating a similar effect of similar modes of life on the two types of shell. It may be worth while here to emphasise the fact that nautiloids are entirely unknown from the Rhaetic, a period that has generally been described as almost "fatal" to the Ammonoidea; but, since nautili are abundant again in the Lower Lias it is clear that their apparent eclipse in the Rhaetic is due merely to failure to collect them owing to lack of suitable exposures. Most Triassic and later nautili, however, have simple suture lines and are primitive types, while on the contrary the smooth groups with simple septal edges in the Carboniferous and Permian are globose and perforate, so that we can rest satisfied that the Ammonoidea were not replenished by fresh nautiloid stocks after their first appearance.

IV. "*BACTRITES*" AND RECAPITULATION.

In the opinion of Sandberger, Beyrich, Frech (1902), and others, "*Bactrites*" stood in the same relationship to *Goniatites compressus* as the straight *Baculites* did to certain coiled ammonites. That is to say, these observers were struck by the resemblance of "*Bactrites carinatus*" to *Gyroceratites* (=·"*Mimoceras*") *gracilis*, and the same impression will probably be shared by all who compare actual examples of those two forms. Frech promptly took this one genuine "*Bactrites*" (*B. carinatus* Sandberger = *B. ellipticus* Frech) to be the genotype, but unfortunately the mere mention of an orthoceratid with marginal siphuncle (*B. subconicus*) by Sandberger, although a *nomen nudum* for nine years, seems to make this the type of "*Bactrites*"; and Schindewolf thus created for *B. carinatus* the new genus *Lobobactrites*. This

change may be regrettable but it does not really reflect more than the inadequacy of our nomenclatorial rules. Moreover, the restriction of *Bactrites* to the *subconicus* group makes it a synonym of *Orthoceratites* Blumenbach[1]. No doubt, this has somewhat cleared the air, for there has always been confusion between this "*Bactrites*" and *Lobobactrites*, not simplified by the subsequent discovery of very similar protoconchs in what were believed to be the two groups. Let it be clearly understood then, that there is one group of "*Bactrites*" (now renamed *Lobobactrites*) in which there is pronounced bilateral symmetry and a peculiar dorsal keel, or rather raised band. The ornamentation is very faint ventrally, as in some *Gyroceratites*, but it is more distinct on the two sides of the dorsal band, and strongly projected backwards, like the septal edges. This whorl shape and ornamentation closely agree with *Eubaculites vagina* (Forbes) in which, however, the siphuncle is on the opposite side. There is no resemblance of this ornamentation to that of forms like *Kokenia obliquecostata* Holzapfel, which Correns has now shown to be a nautiloid; and even such angulate orthocones as *Gonioceras* are not in the least comparable. On the other hand there are the so-called "*Bactrites*" of the *subconicus* group (*Orthoceratites*) in the Wissenbach *Orthoceras*-Slate which have a straight septal edge, the siphuncle often not quite marginal, and the general aspect of *Orthoceras*, *i.e.* concave septa and a more or less circular cross-section. And all the supposed "*Bactrites*" protoconchs hitherto found may belong to this group, *i.e.* to *Orthoceratites*.

Now it might be asked whether these differences are really sufficient to separate *Lobobactrites*, as presumably an uncoiled goniatite, from the orthoceratitid "*Bactrites*," *i.e.* a nautiloid. In this connection it must be remembered that these straight forms lived together in exactly the same environment and that the orthocone shell reflects nothing more than adaptation to a similar mode of life. But apart from this functional resemblance the convergence is accentuated by direct relationship; for it has been shown above that the early goniatites are still closely related to the Nautiloidea and we know from the occurrence of genera like the Triassic *Rhabdoceras*, the Jurassic *Acuariceras* and the various Cretaceous baculitid forms that the possibilities of variation are extremely limited, even after the suture line has taken on frilling. Let it also be noted that there are no passage forms between the straight *Rhabdoceras* and the spiral *Polycyclus*, between *Acuariceras* and the coiled *Spiroceras* or *Strenoceras*, a point of some importance. For Hyatt considered that those who sought to claim that *Bactrites* was a "degraded" form of "*Mimoceras*" should produce forms of the latter, and of the original *Anarcestes*, in which the adults were uncoiled, after a close-coiled stage of growth had been passed through. Hyatt went on to say that such "degraded forms" were common in the Jurassic and Cretaceous, and enabled the observer to connect *Baculites* with the normal coiled ammonoids of the same formations; but he could not have named, and it is impossible at the present day to name, definitely and not just vaguely, such transitional types because they do not exist.

[1] Genotype. *O. gracilis* Blumenbach, *Specimen Archaeologiae Telluris*, 1803, p. 21, tab. ii, fig. 6, a species with almost marginal siphuncle.

It will be seen that Hyatt relied on *post hoc ergo propter hoc* evidence. In the case, however, of a Cretaceous form like *Myloceras* (Spath, 1925, Pl. XXXV, fig. 2) the coiling of the inner whorls may be loose, and even helicoid, before it becomes ammonitic, to uncoil again in a straight shaft and the final crozier of the body chamber. The same occurs in *Scaphites*, as mentioned above (Fig. 4), or in *Tropaeum* and its allies, and, as in *Cicatrites*, even tuberculation may be developed in these Cretaceous lytoceratid offshoots suddenly and not, in accordance with the orthodox view, first on the body chamber, and then spreading to successively earlier whorls in later species.

On the other hand, ever since *Orthoceras* began to be common, it has always produced some species with a more or less marginal siphuncle. Apart from *Orthoceratites*, others of these have been given generic rank, *e.g. Protobactrites* Hyatt, created for *Orthoceras styloideum* Barrande, of the Silurian, with external siphuncle. But *Protobactrites*, in spite of its suggestive name, also is only an *Orthoceras*; and the meaningless diagnosis (Hyatt, 1900, p. 518) led Lang (1919, p. 55) to write that "it was implied, though not explicitly stated that *Protobactrites* also had a protoconch." Now *P. styloideum* and *P. pleurotomum* Barrande (1868, Pl. CCXCVI, figs. 1–24), happen to be truncated species, the latter of which even repaired its apex after shedding the earlier air chambers, scarcely what would be expected in the ancestor of ammonoids. Moreover the Ordovician *Bactroceras*, with marginal siphuncle, is really much more like the Devonian "*Bactrites*" than is *Protobactrites*. Besides, there are the Upper Carboniferous *Orthoceras carbonarium* (J. Perrin Smith) and the Permian *O. paternoi* or *O. adrianense* Gemmellaro, which have marginal siphuncles, yet are inseparable from the contemporary true *Orthoceras*. It is important to insist that although some *Orthoceras* existed in the Ordovician, the narrow-siphuncled forms did not become common until the Gothlandian, and they are relatively far more abundant in the Devonian than the coiled nautiloids, even the loosely coiled "*Gyroceras*," the commonest spiral form among them, being called "a rarity" by Barrande. In the Carboniferous Limestone, as every geologist knows, as well as in the marine Upper Carboniferous, such as the Cisco Formation of Texas, straight shells are largely predominant, and it is important to note that all these rich *Orthoceras* faunas have so-called "*Bactrites*," *i.e.* extreme offshoots with the siphuncle marginal, not central. Some of these have a swollen protoconch, but so does a coiled nautiloid of the Trias for no obvious reason; and no one ever suggested that these started new stocks of ammonites. Again in the Lower Permian of Sicily, *Orthoceras* is the dominant nautiloid, with other "*Bactrites*" forms. Finally, among some 4000 cephalopods from the Middle Trias of Bosnia and Montenegro, recently acquired by the British Museum, there is still a preponderance of the straight nautiloids over the coiled ones in the proportion of at least ten to one.

Now considering the abundance of *Orthoceras* throughout the later Palaeozoic and the Mediterranean Triassic, it does not seem probable that the forms of the Middle Devonian *subconicus* group owed their *Orthoceras* aspect merely to convergence. They were simply members of the dominant and commonest cephalopod stock and differed merely in the position of the siphuncle, not a generic character

in other nautiloids and not even specific in other "*Orthoceras.*" Conversely *Lobo-bactrites*, by various features and especially by its suture line and septal surface, shows what Hyatt and others called "close affinity" with *Gyroceratites gracilis* (= "*Mimoceras*" *compressum*) and thence the goniatites. So that, allowing for the more pronounced difference in the septal edges, "*Bactrites*" and *Lobobactrites* (with unknown beginning?) are probably as distantly related as are *Rhabdoceras* and an Upper Triassic *Orthoceras*. The view that evolution was necessarily from straight to coiled, already shown to be contrary to the evidence, was, however, based on the supposed recapitulation of an ancestral orthocone stage by the young of one spiruliform goniatite, namely *Gyroceratites* (= "*Mimoceras*") *fecundus*. This view, of course, seems to me to be entirely untenable, and since I have elsewhere discussed in full the unreliability of such recapitulatorial evidence it must suffice to state that it is not theoretical considerations but practical experience that has taught me to disregard it. On the contrary I am now accepting it as a matter of course that in ammonites at least ontogeny is not an epitome of phylogeny and that new cha-racters appear in the young and only afterwards encroach on the later whorls.

Without going into all the evidence again, I may briefly recall that, as uncoiling in the Cretaceous genera mentioned above began on the inner whorls, so the keel in *Quenstedioceras* and other cadoceratids or the groove in *Schlotheimia* and in *Berriasella* first appeared in the young; and the spines of *Asteroceras* or the run-cinate periphery of *Kepplerites* were caenogenetic, *i.e.* they appeared as new and not inherited characters in the early stages and were fully developed and persisted to the last whorl only in the geologically later species of these genera. It is clear that if in *Gastrioceras* the tubercles appear (caenogenetically) to support the depressed early whorls, and the same strengthening device is adopted by many a later stock, the mere possession, by a given shell, of coronate inner whorls is no indication of derivation from a coronate ancestor. The same applies to all the other characters of the shell and even to the septal edge.

We have been taught to deduce descent from the individual development of the ·suture line and although I have myself used such recapitulatorial evidence, I now consider the recognition of so many goniatitic stages in, for example, a Cretaceous ammonite to be as meaningless as is the hypothetical skipping of some of these stages in other Cretaceous forms, for the suture line of all ammonites, during growth, has to modify by degrees. Looking at the ontogenetic development of the suture line in *Pseudoceratites*, *e.g.* the engonoceratids, recently (1931, p. 339) discussed, I do not expect to see arrestation at an imaginary ceratitic stage any more than retro-gression but merely modification of a hoplitid suture line, comparable to that of *Anahoplites* though of a different type and of course appearing as soon as mechanical considerations necessitate or allow. This flat and smooth form, with its asymmetry and extraordinarily long range undoubtedly led a mode of life different from that of the other ornamented but short-lived hoplitids, and engonoceratids were similar adaptations. Another Gault form, *Mojsisovicsia*, was described by Hyatt (1903, p. 25) as probably a "Cretaceous member of an [unknown] primitive stock which began with *Psiloceras* in the Jurassic." After a lifetime spent in elucidating the laws of

development in ammonites, Hyatt was completely puzzled; yet from simple morphological considerations the affinity of this genus with the contemporary keeled *Dipoloceras* is obvious.

This does not imply that the inner whorls, after the early smooth stage, are always useless for revealing genetic affinity, for the adult may be highly modified; for instance in "*Nautilus*" *subcarinatus* Young and Bird, it is only the earlier volutions that show the *Hildoceras* characters of the ancestral stock. As in the slowly evolving *Ptychophylloceras* of the Middle and Upper Jurassic and even Lower Cretaceous, for example, this is normal development, and it is probable that a change in the mode of life of the adult, perhaps with the mantle reaching over the shell in many forms, caused loss of ornamentation or a modification of the body chamber. In other words the inner whorls of such modified forms, changing with growth like all ammonite shells, do not "recapitulate" an ancestral form, but merely show the characters of the group to which its unmodified congeners belong.

Uncoiling may begin on the outer whorl, as Hyatt held, but forms like *Crioceras eryon* and other Lower Lias ammonites illustrated by Reynès (1879, Pls. VIII, XIX, XLI, etc.), or the evolute *Arnioceras* figured by Quenstedt (1883, Pl. XIII) and Lange (1932, Pl. VII), if not merely pathological or due to the growth of serpulids, at least never produced uncoiled offspring. Conversely the Triassic *Choristoceras* is not the ancestor of the straight *Rhabdoceras*, with its shell thin on the dorsal side (Fig. 5*h*); the separation of the outer whorls in some individuals of *Choristoceras* signifies possibly still less than the modification of the body chamber in *Macroscaphites*, as compared with the uncoiled *Costidiscus*, a difference that has been attributed merely to sex. *Choristoceras* was clearly an adaptation to a special mode of life and not a senile but a flourishing stock, although it disappeared during the general eclipse of the ammonoids in the Rhaetic.

The "laws" that Hyatt postulated to my mind are not natural laws, and his "Genesis of the Arietidae" is to me one huge fallacy, in so far as it is based on the recapitulation of a smooth ancestor. If Devonian goniatites at first had imperfectly closed umbilici, this is only what would be expected in forms still so near to the ancestral nautilicones. Eichenberg (1931) has lately described from the Lower Devonian not only "*Mimoceras*" and *Anarcestes*, but two new genera *Mimagoniatites* and *Mimosphinctes*. The last, like Barrande's *Goniatites lituus* (= *Palaeogoniatites* Hyatt), however suggestive, is too incompletely known to be discussed, but they may all have umbilical perforations. Yet this author ascribes to *Mimagoniatites bohemicus* (Barrande) forms with mimoceratoid inner whorls, whereas some true Bohemian (Hlobučep) examples of this species that I have sectioned show only a kink of the first whorl, but no opening. Moreover, in his *Mimagoniatites*, Eichenberg has observed that the two or three chambers following on the protoconch were still large and that narrowing set in only gradually, so that the smallest cross-section was about a third of a whorl away from the protoconch. This does not seem to favour derivation from "*Bactrites*," but it is probably not constant and, in any case, in the more closely coiled *Anarcestes* the early stage may still be variable (Fig. 2),

while Sandberger's (1851) figures show that even the Upper Devonian *Gephuroceras* and *Manticoceras* may be perforate.

Yet *Anarcestes* represents a progressive stock and if there is any connection between *Lobobactrites* and *Gyroceratites* (or "*Mimoceras*") then it represents a "degenerate" branch, originating in a form with very unstable inner whorls, as clymenids arose from a similar group in which the position of the siphuncle was unstable. *Lobobactrites* appears too late to be a radicle of the Ammonoidea and the orthoceratid "*Bactrites*" that existed from the Ordovician to the Permian never showed any coiling, except, possibly, a curved apex, like the specialised *Poterioceras*. Moreover, an involute and oxycone form like *Pinacites*, existing already in the Lower Devonian [not to mention the still earlier Silurian *Agoniatites* described by Denckmann (1900) and the Eodevonian *Tornoceras* and *Epitornoceras* recorded by Frech (1902)] clearly shows that the tendency to uncoil (in the Lower Meso-devonian) was preceded by a long period of more and more complete incoiling of the Ammonoidea. All the Middle and Upper Devonian *Anarcestes*, *Agoniatites*, and *Manticocerates* which I have examined were closely coiled, with only sometimes a kink in the first whorl (Fig. 2*f*); and while there is no reason to doubt that such perforate individuals as have been figured by Sandberger (1851), Holzapfel (1895), and others, did exist, the tendency to ever closer coiling or greater involution be-comes evident in the Carboniferous. Like the modification of the protoconch from asellate to latisellate and then angustisellate so the gradual incoiling was slow, com-pared with the rapidity of uncoiling in the aberrant offshoots.

V. THE PRIMITIVE CEPHALOPODA.

It is desirable to give a brief review of the principal types composing the earliest cephalopod faunas if a picture of the development of the class as a whole is to be obtained. The great majority of the early curved and straight forms had a large siphuncle which often contained part of the viscera as is evidenced by the muscle impressions. Some had an apical cone (Fig. 13*a*) which was either short and strongly inflated (*Nanno*) or long and tapering (*Proterocameroceras*), and Grabau recently (1929) again echoed the opinion of Ruedemann (1905) that these forms which at first had only a siphuncle and no camerae (*i.e.* air chambers), were the most primitive of the holochoanites.

Now Hyatt did not see in the large, inflated, apical cone of *Nanno* more than a primitive character of the nepionic stage such as may be retained in various genera; and it is to be noted that *Nanno* (Middle Ordovician) is later even than the Canadian (Tremadocian) *Piloceras* without pre-septal cone and with the siphuncle either in contact with the ventral wall of the conch or subcentral. But the primitive *Piloceras* itself is preceded by Ozarkian forms some of which have wide marginal or sub-marginal siphuncles and a curved cup-like shape (*Clarkoceras*), while others are more elongated and have a narrower siphuncle (*Ellesmereoceras*) or are fusiform, short and inflated (*Eremoceras*). They are all densiseptate (Fig. 12), often laterally compressed, and the marginal siphuncles are holochoanitic, *i.e.* the prolongations

of the septal funnels penetrate the preceding septa but there are no endosipho-sheaths. These are developed only in the next higher, Canadian, formations where long orthocones also first appear although no *Orthoceras*.

Unfortunately, correlation of the deposits of different areas is as yet far from accurate and there is room for far more between the Cambrian and the Ordovician than many English geologists realise. But meanwhile we may take it that the numerous cephalopods said to exist in Ozarkian beds (although very inadequately described up to the present) agree on the whole with the Tremadocian (Lower Canadian) fauna but differ considerably from that of the Ordovician above. Foerste, in fact, speaks of the interval between the Canadian and the Ordovician as one of
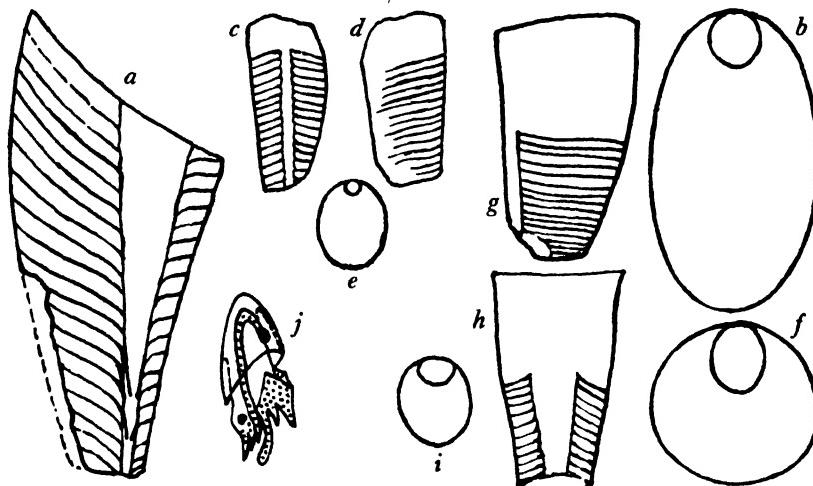

Fig. 12 a–i. Ozarkian Cephalopoda. *a, Clarkoceras newton-winchelli* (Clarke) after Ruedemann (1905) ∧ 1½. *b, Clarkoceras holtedahli*, Foerste (1921). *c–e, Ellesmereoceras scheii*, Foerste (1921). *f, Ellesmereoceras robsonensis*, Walcott (1924). *g–i, Eremoceras syphax* (Billings) after Foerste, 1921. *j*, diagrammatic original cephalopod, after Ray Lankester (copied from Ruedemann, 1905).

the great time-breaks of geology. Shumard's descriptions (1863) of the doubtful "*Orthoceras*" *ozarkense* and the "somewhat abundant" *Lituites complanata*, a coiled shell, do not, however, indicate more primitive types than those of the Magnesian Limestones of Newfoundland (Canadian Quebec Group) and Scotland (Durness and Skye).

Clarkoceras, of Upper Ozarkian age, is a particularly interesting genus among the arcuate forms of the fundamental family Piloceratidae (and "Cyrtendocera-tidae," as emended by Foerste). According to Ruedemann (1905, p. 337) the "entire endosiphuncular structure was distinctly in a process of dissolution, resulting from the reduction of the size of the siphuncle in consequence of the more complete withdrawal of the visceral cone." But as *Clarkoceras* is early and is holochoanitic, at least in the Ozarkian, it denotes a more primitive stage than *Piloceras*. This is probably another case of a new character first appearing in the

early stages, and in my opinion the filling of the siphuncle with endosipho-sheaths is a secondary feature as much as the apical cone of *Nanno* (Fig. 13).

The early stages of a Tremadocian *Cyrtendoceras* or *Cyrtocerina* already essentially resemble the shell apex of the living *Nautilus pompilius*. From this time onwards the nautilid stock is found continuously. We can, with Dacqué (1921), recognise only a very slow progress during the Palaeozoic towards greater involution, but I do not believe that there is any difference between the mode of life of the recent forms and that of their "swimming ancestors." The shell, apart from its function as a protection for the soft parts, is clearly a swimming organ; and we have already seen that bilateral symmetry is common to all young nautiloids. As with other characters I consider it probable that the coiling was first acquired in the young and not in the adult; the young of the ancestral forms with arcuate shells
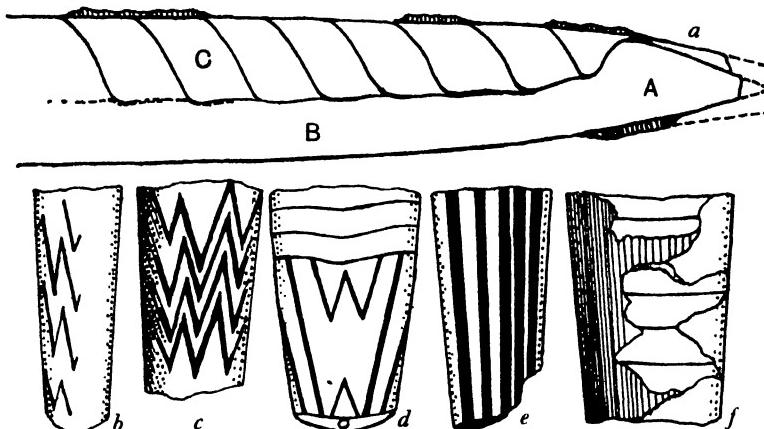

Fig. 13. Straight Nautiloidea. *a, Nanno belemnitiformis* (Holm), Ordovician, with pre-septal apical cone (*A*), wide marginal siphuncle (*B*), and camerae (*C*). After Holm. *b–f*, various Ordovician to Devonian orthocones, showing asymmetrical colour markings. After Foerste, slightly enlarged.

that found themselves provided with considerable curvature may be assumed to have led a more freely swimming existence than their less curved kindred; but all the intermediate types co-existed from the earliest period onward and the straight and late *Orthoceras*, often with colour-markings (Fig. 13) as in other shallow-water Mollusca, is as much an adaptive development of the common ancestor as is the regularly or irregularly coiled nautilid shell.

The small and incompletely known *Cyrtoceras cambria* Walcott (1913, p. 98, Pl. VI, figs. 4, 4 *a–c*) from the Upper Cambrian of China, also apparently resembles Canadian forms; and since all the Ozarkian nautiloids are holochoanites, there seems to be no reason for Teichert's (1929) assumption that it is the ancestral form of the "cyrtochoanites," *i.e.* the polyphyletic actinoceratoids of the table on p. 455. No coiled nautilid comparable to Shumard's *Lituites complanata* seems to have been rediscovered in association with the primitive curved and cup-shaped forms cited above. But if Hyatt were right in considering a loosened body chamber or free outer

whorl to represent the beginning of uncoiling this Ozarkian lituitid would be already a degenerate type. Others have looked upon *Lituites* as a stage in progressive coiling, but I consider it to be an adaptation to a special mode of life in the adult, *i.e.* the early nautilicone, free-swimming, stage was succeeded by a mode of life comparable to that of some of the straight types here discussed. The loosened apertures of some of the imperfect lituitids, like comparable terminations in, for example, *Ophidioceras*, *Trochoceras*, etc., often with constricted mouth border, of course represent still different modes of existence. In any case, whether coiled forms already existed side by side with the curved and straight forms or not, it would be but a short step from *Piloceras* or a similar cyrtocone to the ancestral open cup or capulicone (see Fig. 12*j*).

There will still be little opposition to the view of Hyatt (1894, pp. 360-1)—quite independent of recapitulation—that all the radicle forms of Cephalopoda and the shell-covered forms of other kinds which have the protoconch, namely the Gastropoda, *Tentaculites* and the ancient Pteropoda, probably had a common origin in some chamberless and septaless form similar to the protoconch or to the apical cone. It is assumed that the primarily benthonic archetype, with calcareous shell, *i.e.* the first shell likely to be preserved as a fossil, was shaped like an open cup, and by the differentiation of the visceral sac air chambers were formed and the siphuncle produced. The formation of greatly enlarged funnels, of endosipho-sheaths and so forth, combined with gigantism, etc., that characterised the later endoceratids, is clearly a secondary modification. It is the early holochoanites (ellesmereoceratids) that may be considered to be the ancestors of both the endoceratids and the ortho-ceratids (and their actinosiphonate offshoots), but the more active contemporaries, especially those with greater curvature, *i.e.* the early Nautilidae, adapting themselves to a different mode of life after the early naked stage never required a loaded siphuncle.

Now all this represents little advance on what was known many years ago, but the genetic picture has since been distorted by the stressing of the "straightness" of the early forms in deference to a hypothetical cycle, and by the plausibility of the "reconstructions" of *Volborthella*.

VI. THE SUPPOSED CEPHALOPOD *VOLBORTHELLA*.

The Lower Cambrian *Volborthella* (Schmidt, F., 1888) has been claimed to be the earliest cephalopod. It may be questioned, however, whether the evidence justifies the assurance with which this claim is pressed by some. *Volborthella* was first described from the so-called "Blue Clay" of Esthonia in which microscopic ptero-pods [Hyolithes] also make their first appearance, and it is possibly identical with Billings's genus *Salterella* (see Clark, 1924, p 8), also formerly a "pteropod." The slabs of rock with *Volborthella* in the British Museum are covered with many hundreds of specimens of these minute cones, but they are the most unconvincing "cephalopods" that could be imagined. Schindewolf (1928, p. 68) is of opinion that it is a real cephalopod and a primitive representative of the "Orthochoanites."

I disagree entirely and with Pompeckj (1928, p. 87) I question Schindewolf's deductions as to the phylogeny of the earliest nautiloids.

The little cones are so variable and so irregular, and their preservation is so different from that of the later and larger cephalopods with hydrostatic shells, that with Krause and Gürich (1928, p. 88) I cannot accept them as cephalopods. Hyatt (1900, p. 516), ascribing to *Volborthella* not only conical septa but a "flaring" living chamber, in any case, had quite a different opinion, for he regarded it as one of a highly specialised family Ascoceratidae, of the sub-order Mixochoanites, animals adapted to a peculiar mode of life in the adult and certainly without the slightest affinity with, or even resemblance to, *Volborthella*. This was recognised by Foerste (1925, p. 4), who stated that *Volborthella* should be associated with *Salterella*, without, however, expressing any opinion as to its cephalopod (or pteropod) nature.

Karpinsky (1903) figured a number of specimens of *Volborthella* of varying shapes, but all his illustrations are greatly enlarged, and those who do not know the actual fossils may easily take certain features, as, for example, his diagrammatic drawing of the supposed cast of an air chamber (Fig. 6, enlarged × 20), as proving the orthoceratid nature of this fossil. The same applies to Clark's restoration of *Salterella conulata* (1924, Pl. II, fig. 10), and to Schindewolf's diagrammatic section of *Volborthella tenuis* (1928, p. 70, Text-fig. 1) or his photographs (1929, p. 175). The appearance of the "siphuncle" (or rod, or columella) as represented in Karpinsky's Fig. 3 (p. 35) is not unusual, but in many instances the septal surfaces resemble his Fig. 7 (No. 5), and it is not at all likely that they are always the "apertures." Again, in the material seen by me, septation (or tabulation or whatever else it may represent) is irregular or altogether absent and in any case merely a concentration of black dust particles; nor is it clearly visible in any of the ten picked casts figured by Karpinsky, except possibly in Fig. 2, although even in that the distances between the "septa" are not regular. This irregularity is also shown in Clark's type (Pl. II, fig. 8). The transverse striation, again, is more like that of certain rugose corals than of molluscan shells; and the preservation of these sandstone casts, washed together in great numbers (like other pteropod oozes) is entirely different from what might be expected to remain of fragile, soft, and incompletely calcified cephalopod shells after becoming imbedded in a sandy matrix. On the other hand it might be argued that the absence of the early septate portion (and, of course, of the initial chamber) and the impossibility of examining the structure of the central "siphuncle," are due to this poor preservation in a rough, sandy matrix; but the examination of many sections under the microscope is most unconvincing, although what might have been septation, sometimes conical, was seen in a few instances, and others showed a central rod, or rather stain, that could be taken to be a siphuncle. There is certainly nothing like the appearance of the supposed septa figured by Clark (1924, Figs. 5, 6), even if there is external resemblance to his figures. That is to say that, while *Volborthella* has a *Styliola*-like cone of very variable shape, what is known of its internal structure does not seem to warrant the restorations which have been made or its description as orthochoanitic, nor could it be considered as indicating a hydrostatic apparatus. There may have been an

abortive attempt of some primitive pteropod stock at septation or tabulation, but it was almost certainly independent of the true cephalopod ancestor.

It is necessary, then, to keep an open mind regarding the nature of *Volborthella* and *Salterella*. Ulrich's (1911, p. 503) assumption that cephalopods and gastropods "originated in oceanic basins to the south of the Mississippi embayment" seems as premature as Grabau's (1929) attempt to give "provisional" names to problematical groups, but since this author and others like Dacqué (1921) and Teichert (1929) have definitely accepted *Volborthella* as the primitive ("orthochoanitic") cephalopod ancestor, it is necessary to raise this protest.

VII. THE PHYLOGENY OF THE CEPHALOPODA.

Ignoring, then, the doubtful *Volborthella* (or *Salterella*) it is found that the earliest cephalopods are holochoanites, without endosiphuncular structures, and that they include no orthochoanites or cyrtochoanites. Future discoveries no doubt will show that these artificial divisions cannot be upheld, but I agree with Troedsson (1926) that the orthochoanites are derived from the primitive holochoanites, *i.e.* the endoceratids (and piloceratids), including *Ellesmereoceras* and *Clarkoceras*. A tabular scheme to show this interrelationship is now given, but for the sake of clearness many of the smaller groups are omitted.

Table I. *The early Nautiloidea.*
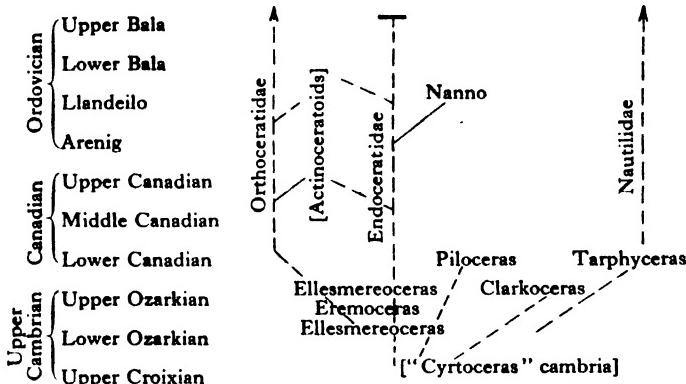
It should be added that there is no natural dividing line between cyrtocones and gyrocones; as the result of normal growth in these tubular organisms one would expect to find all intermediate stages. Also the coiled forms of the sub-families Tarphyceratinae, Trocholitinae, and Lituitinae, found already in the Tremadocian, present a great wealth of shapes and coiling. They became specialised in almost every direction and gave rise to uncoiled shells, sometimes with cast-off air chambers, and this at the period of maximum development of the straight-shelled endoceratids. It is clear that secondarily straight shells like the Ordovician and Devonian jovellanids must be attached to those curved and coiled contemporaries with which

all the other characters, including the siphuncle, show them to be related; but as already mentioned a classification of these cephalopods into distinct sub-orders by the structure of the siphuncle alone must be unnatural. The ammonoid *Cyrto-clymenia* and the Tertiary nautiloid *Aturia*, with dorsal siphuncles, developed long siphonal funnels quite independently and have no affinity whatever with the endoceratids. Conversely some endoceratids like *Protocycloceras* may become ortho-choanoidal without being distinguishable specifically from the typical holochoanoidal forms. Only close association in date of the various forms and consideration of all their characters, even microscopic, will eventually lead to a more natural classification, but it will not be a return to a system based on coiling or whorl shape, as might be wrongly suggested by the accompanying table I or the writer's (1927*a*, *b*) sub-division of the Mesozoic nautili.

In 1894, Hyatt (p. 365) admitted that "the whole series of forms from the straight to the nautilian, were present in the earliest period." But elsewhere he asserted that Diphragmida and Endoceratida slightly preceded the nautilian forms or at least that their earlier existence could be deduced by "noting how the number of coiled shells diminished as the Palaeozoic was followed backwards, especially when the lost records of Protozoic time were taken into account" (p. 366). Later (1900, p. 533) Hyatt again attempted to prove statistically that in the earlier formations, orthocones, together with their almost invariably associated cyrtocones, predominated, and he went on to say that "the Permian had but one surviving family of orthocones and four of the coiled groups; in the Trias the ratio was one to six, and in the Jurassic coiled forms alone persisted. Thus a slowly working tendency was apparent, leading to the production of more and more closely coiled cones and the elimination of straight and slightly curved forms."

Now it is a matter of common observation that the straight shells are most abundant still in the Upper Palaeozoic, but when even Blake (1892, p. 285) who had monographed the early British cephalopods, uncritically repeated Hyatt's dictum that in the Palaeozoic periods we reached in succession the orthoceratitic, the cyrto-ceratitic, the gyroceratitic, and the close-coiled nautiloid stages, general palaeontologists like Dollo (1922) may be forgiven for accepting this as a true evolutionary series. It will be seen, however, that there is neither gradual elimination of the straight forms which indeed are far more numerous than coiled nautiloids throughout the Permian and Triassic, nor is there any successive replacement of these straight shells in time by cyrtocones, gyrocones and nautilicones. Otherwise Hyatt's remarks only show that the straight type of nautiloid shell co-existed with the truly nautilian forms from the Ozarkian onwards; and if one can recognise replacement at all in this connection one should look for it in the Dibranchiata, like the Triassic *Atractites*, *Aulacoceras* and the host of Jurassic and Cretaceous Belemnitidae that continue the "straight" development.

Since Quenstedt's days such successful recent cephalopods as cuttle fishes and squids have been universally considered to have descended from the belemnitids; and I agree with Hyatt (1894, p. 352) and von Bülow (1915, p. 23) that belemnitids arose from the *Orthoceras* stock by inclusion (and by resorption) of the shell within

the folds of the mantle. We thus have a host of descendants of the straight nautiloids, still flourishing as the most highly organised and largest of our Mollusca, whilst the single, coiled, genus *Nautilus* has lingered in undistinguished isolation at least since Miocene times.

The following genealogical tree of the Cephalopoda attempts to illustrate these interrelations.

Table II. *The evolution of the Cephalopoda.*

Recent	Dibranchiata	Tetrabranchiata Nautilus	(?)
Tertiary			
Cretaceous	Belemnoidea	Nautilidae	Baculites
Jurassic			Acuariceras
Triassic			Rhabdoceras
Permian	Orthoceratidae		Ammonoidea
Carboniferous		Cyrtoceras	
Devonian		Gyroceras	Clymenia
Silurian			Lobobactrites
Ordovician	Endoceratidae Nautilidae	Lituites	
Tremadocian			
Cambrian			
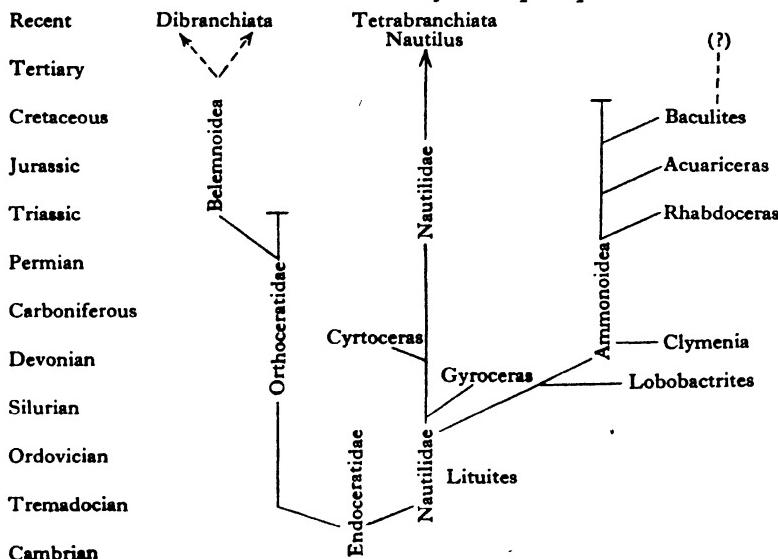
VIII. THE DIBRANCHIATA.

I can offer no new facts regarding the evolution of the Dibranchiata and take this opportunity only to make a suggestion in connection with the sudden disappearance of the Ammonoidea at the top of the Cretaceous. Of course, Steinmann's view of a possible connection of the argonautids with the ammonites has been rejected before and the octopods may have been derived from an early decapod type (perhaps the Triassic aulacoceratids) before the three principal decapod stocks (Belemnoidea, Sepioidea, and Teuthoidea) had become distinct, as Naef suggested (1922). I have no doubt, after all that has been said concerning the close affinity between ammonoids and nautiloids, that a Cretaceous form like *Palaeoctopus* and a *Scaphites* (which Steinmann cited in particular) have not the slightest affinity. But the view that at least some of the present-day dibranchiates represent the shell-less descendants of an ammonite stock is not so impossible as is generally believed; for example, the octopods are an extremely varied group and the interrelations of the different sub-orders with *Palaeoctopus* are still very obscure (Robson, 1932).

It is believed that the earliest dibranchiates arose from a straight (and presumably tetrabranchiate) nautiloid (*Orthoceras*) in consequence of inclusion of the shell

by the mantle, and this process was not gradual considering the sudden appearance of the aulacoceratids and the extraordinarily rapid rise of the Belemnoidea.

It is not probable that such a process recurred in the history of the Cephalopoda, but even if it did it would probably not have proceeded on exactly the same lines. Thus it does not follow that a resistant phragmocone, with or without solid guard, would again have been formed. If inclusion by the mantle and consequent resorption of a thin and fragile baculite shell be envisaged, the early initial coiling might conceivably be held to be an obstacle, although it is not certain that, for example, the Cenomanian *Cyrtocheilus* (an early baculite) had a coiled beginning like some Senonian species described from American deposits, and in any case the early coil is microscopic even in many Albian hamitids, unlike the coiled phragmocone of a *Spirulirostra*.

However, there need not have been absorption but mere discarding of the shell. It is known that some species of *Orthoceras* and other nautiloids shed all or part of the earlier, camerate, portion and sealed off the posterior end by secondary deposits. This was a first step towards the suppression of the shell, and in any case it was no longer a hydrostatic apparatus. The ascoceratids even formed two entirely different types of shell in their ontogeny. The origin of the belemnites is quite independent of these early attempts, but it seems to me conceivable that in any heteromorph or uncoiling stock the shell may have been shed in the young in order to enable the animal to move about more freely. I do not consider the slight complication of the septal edge to be decisive evidence against this view. There was no orthogenetic tendency in the ammonoids to complicate the suture line, throughout the Mesozoic, as has been asserted; when the whorl section of a baculite or hamite was circular, the septal edge generally simplified and the hold of the animal upon its shell was greatly reduced. The complex suture line was never useless in the ammonoids any more than the guard of the belemnites; and the suggestion that they merely reflect an ingenious way of depositing superfluous calcium carbonate (Lang, 1919, p. 64) seems to me quite untenable.

Baculitids were the dominant ammonoid stock at the end of the Cretaceous. There are many baculite limestones as crowded with these straight shells as any belemnite "battle field," especially in the Maestrichtian, the highest ammonitiferous formation of the Mesozoic. But the maximum in size was attained already in the Campanian. True ammonites still occurred together with the highest baculites and a few other heteromorphs; and *Indoceras baluchistanense*, the latest known ammonite species, with seventy-five lobes and saddles in its suture line, shows no "senile" characters, but recalls rather the acme of ammonite specialisation in the Upper Trias. But these ammonites had become very scarce, all the same, in comparison with the straight forms, and the increasing competition by the naked decapods may well have prompted some admittedly very adaptable heteromorph stock to attempt a shell-less mode of life. The hydrostatic portion of a large *Baculites grandis*, of course, is still nearly half of the total shell, but in similar smaller species it may have been reduced or it may have been shed at an early stage. In any case no passage forms are known between *Orthoceras* and *Atractites* or *Aulacoceras*. We know now

that new types appeared as saltations and that it would be as idle to insist on seeing a complete series of passage forms from *Orthoceras* to the belemnites, as a progressive modification of the shell in a baculite or other hamitid. It is not in the least surprising that there are no passage forms as envisaged by John (1909, p. 14), with gradual reduction of the shell muscles, until there was finally no formation of air chambers or of a pearly layer; such links cannot be demonstrated even with progressive series that merely modify the shell. Nor could the occurrence of a similar phenomenon in an earlier stock, *e.g.* the Callovian *Aduariceras*, be proved, another slender and extremely fragile shell, except possibly by the discovery of numbers of exceedingly well-preserved impressions in a very fine-grained shale.

It does not seem necessary to me, however, to stipulate a frequent repetition of this process. Conditions were entirely different at the end of the Cretaceous, with true ammonites known to be going down before the baculitids. If their shell-less descendants were assumed to be more virile than the last few surviving belemnites, it would be obvious why the latter also almost disappeared about the same time. In the economy of nature the disappearance of the shell of baculitids would have made as little difference as the replacement of *Orthoceras* by belemnitids, but of course the apparent difference in the late Cretaceous and early Tertiary marine faunas in Europe is enhanced by an accidental absence of really transitional formations.

IX. SUMMARY.

The principal conclusions arrived at may be briefly (though perhaps somewhat too bluntly) summarised as follows:

1. *Volborthella* (or *Salterella*) is not a cephalopod (? pteropod or so-called pteropod).

2. The ancestral cephalopod was a bilaterally symmetrical cyrtocone or capulicone, with wide and unloaded marginal siphuncle (holochoanitic) and no apical conch (or pre-septal cone). (But see Addendum, p. 462.)

3. Orthocones and nautilicones (at first, ophiocones) developed independently from the primitive cyrtocone. The stages of progressive coiling do not represent an evolutionary series.

4. Ophiocones with more or less imperforate umbilicus existed already in the Tremadocian.

5. The protoconch in nautiloids was always calcareous and was never shed.

6. Most gyrocones and cyrtocones (and some orthocones) were uncoiled nautilicones or special adaptations (*Phragmoceras, Ascoceras,* etc.).

7. "*Bactrites*" (a synonym of *Orthoceratites*) is merely an *Orthoceras* with a marginal siphuncle.

8. The earliest goniatites were essentially like the more or less imperforate nautilicones except in the position of the siphuncle, which, however, was notoriously unstable.

9. The Ammonoidea, like the Nautiloidea, were monophyletic and both are far more homogeneous groups than the multiplication of generic names suggests.

10. Goniatites with unstable siphuncle gave rise to the clymenids which (like the holochoanitic nautiloid *Aturia*) died out completely.

11. Ammonites are the descendants of goniatites, not of clymenids.

12. *Gyroceratites* (= "*Mimoceras*") and *Lobobactrites* are derivatives of goniatites.

13. No series from straight to coiled are known but only such as go in the reverse direction, in Nautiloidea as in Ammonoidea.

14. The position of the siphuncle in the nepionic stage in ammonites was extremely variable and has no phylogenetic significance.

15. Palingenetic methods are discredited as the result of the writer's practical experience. Hyatt's and his followers' evolutionary series being constructed on the principle of recapitulation cannot, of course, be used in support of such palingenesis.

16. All characters must be used as a basis for classification and all are variable. Reliance on siphuncular structure only in nautiloids leads to unnatural groupings as much as the undue stressing of the suture line in Ammonoidea.

17. The possibility of *Baculites* having left shell-less descendants is discussed.

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ADDENDUM.

Since writing the above account I have had the privilege of discussing Ozarkian Cephalopoda with Prof. E. O. Ulrich of Washington and it appears not only that the Diphragmida (or endoceratids with tabulae in the siphuncle) must be added to the earliest types listed on p. 455, but that there is a great wealth of new and undescribed genera in the Ozarkian, although '*Lituites*' *complanata* (see p. 452) is of Canadian age.

I. THE BOUND WATER OF PROTEIN SYSTEMS.

### (a) *The hydration of protein molecules.*

ALL the processes of life, as we know it, occur in an aqueous medium, and the biological activity of a tissue is related to the amount of water which it contains (Jordan Lloyd, 1932). The relation of water to the other constituents of the cell is obviously, therefore, of the greatest biological importance. Since the fundamental chemical constituent of protoplasm is protein, the relations of protein or protein-containing systems to water must form the foundation stone of any theory attempting to depict in a general way the relations between water and the living organism. It is now accepted that proteins are colloidal electrolytes and any theory attempting to deal with the balance of water in organisms must also take into account the effect of the non-colloidal electrolytes or salts normally occurring in cells or body fluids. No theory can, of course, be complete that does not also take into consideration the influence of other cell constituents such as lipoids, carbohydrates and so on, but in the present state of our knowledge, an enquiry into the influence of the proteins is probably one from which especial profit will be derived. It will, therefore,

be advantageous to consider first the water balance in protein systems which are not endowed with the properties of life and then afterwards to see how far the same factors are found to affect it in living cells.

Proteins consist chemically of a group of substances characterised by a molecule with an immensely elongated backbone, each unit or segment of which carries a side chain. The backbones are built on a standard pattern, the side chains or R groups vary in their chemical character:

$$
\begin{array}{ccc}
\text{H} & \text{R} \ \ \text{H} & \text{O} \\
\diagdown\ \diagup\ & | & \| \\
\text{C} & \text{N} & \text{C} \\
\diagup\ \diagdown\ \diagup\ \diagdown & \diagup\ \diagdown \\
\text{N} & \text{C}\ \ \ \text{C} \\
| & \| \ \ \diagup \ \diagdown \\
\text{H} & \text{O}\ \text{H} \ \ \ \text{R}
\end{array}
$$
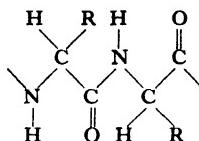
It will be advantageous to consider the possibilities offered both by backbone and side chains for hydration or association with water. These have been considered fully elsewhere (Jordan Lloyd and Phillips, 1933) and will only be summarised briefly on this occasion.

Water can associate with chemical groupings, in which the primary valencies are fully satisfied, by the donation or acceptance of electron pairs. The oxygen atom of the water having two pairs of electrons which are not occupied in covalent linkages between themselves and another atom can donate these to the hydrogen atom of such groups as $-OH$, $-NH_2$, $=NH$, thus forming a hydrated compound by means of a co-ordinate or semipolar link:

$$
-OH \leftarrow O \diagup^{H}_{\diagdown H} \quad ; \quad -N \diagup^{H}_{H \leftarrow O \diagdown H} \ \ H; \quad =N-H \leftarrow O \diagup^{H}_{\diagdown H} \ .
$$
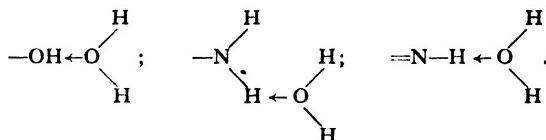
Conversely, the two hydrogen atoms of the water molecule can accept electron pairs from oxygen and nitrogen atoms:

$$
-OH; \quad -N \overset{|}{\underset{H}{-}} H; \quad =N-H; \quad -C \diagup^{O \rightarrow H-O-H}_{\diagdown O-H}
$$
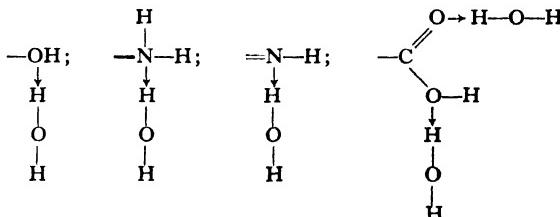
Probably the latter type of co-ordination is the more common. Proteins contain plenty of such groups capable of co-ordinating with water, for instance, gelatin on hydrolysis yields 14·1 per cent. of hydroxyproline; salmin, 7·8 per cent. of hydroxyalanine, better known as serine; and lactalbumin, 10 per cent. of hydroxyglutamic acid. Imino, amino and carboxyl groups are also present in varying amounts, the last two both as uncharged and as charged centres. There is very

little reason to doubt that proteins form hydrates with water just as do many crystalline salts and other crystalline substances such as sucrose. When dry proteins are placed in water, they take up water and swell, with a loss of total volume of the system and an evolution of heat: they behave, in fact, in the same way that sulphuric acid behaves under the same conditions. Sulphuric acid is now recognised to form hydrates with water, and so do most substances which are sufficiently soluble to form strong solutions.

Water held as a hydrate formed by co-ordination of a water molecule with an oxygen- or nitrogen-containing group in the protein molecule, may be called water of imbibition. Nothing is known as to whether one or more than one water molecule becomes linked up at a centre of hydration. Since water molecules co-ordinate with each other, by donation of an electron' pair from the oxygen atom of one water molecule and acceptance by the hydrogen atom of another, it is theoretically possible to moor a whole string of water molecules to one centre of hydration. In the case of crystalline salts forming hydrates in aqueous solution, it is not unknown for one substance to form several hydrates, the controlling factor being temperature. In such a case, it is always found that the lower hydrates are formed at the higher temperature and the higher hydrates at the lower temperature, that is, the lower the temperature, the greater the hydration.

Besides water held by co-ordination at electrically neutral groups, there is also water held at charged centres in the molecule. The terminal groups of the backbone of any one molecule will be an amino group and a carboxyl group respectively and many of the R groups also terminate in the same way. The amino groups are basic in character and exist in water both in the uncharged conditions and carrying a positive charge; the carboxyl groups are acid and also exist in water in the uncharged condition and carrying a negative charge. Proteins exist in solution as zwitterions, that is, both amino and carboxyl groups are charged at the same time (Harris, 1925; Birch and Harris, 1930; Jordan Lloyd, 1933 b). Calling the protein P, proteins in solution may be represented as:

$$
\begin{array}{cc}
^+NH_3 & COO^- \\
& P \\
NH_2 & COOH
\end{array}
$$
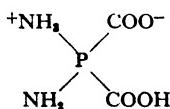
The balance between the charged and uncharged groups depends on the pH value of the system and the ionisation constants of the acidic and basic groups. At the pH of living tissues and body fluids, the protein molecule exists very largely as:

$$
\begin{array}{cc}
^+NH_3 & COO^- \\
& P \\
NH_2 &
\end{array}
$$
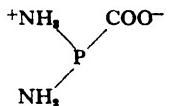
The evidence for this statement has been reviewed elsewhere (Jordan Lloyd, 1933 b).

The point of interest at present is not the actual balance of ionisation but the fact that the protein molecule carries charged centres. Every ion in aqueous solution has round it a shell of oriented water molecules held by the forces of

electrical attraction, and there is no doubt that water is also held in this way round the charged centres of the protein molecule. Again, as in the formation of hydrates by co-ordination to uncharged centres, there is no evidence to show how much water is held at each charged centre. The amount will vary with the concentration of other ions or water-binding centres in the system and with such factors as temperature. Since ionisation increases with temperature, there should be a greater number of charged centres at high temperatures than low ones and, therefore, it might be anticipated that more water will become bound to the protein as the temperature rises, but since the amount of water held at each centre will become less as the temperature rises, the actual hydration due to orientation of water round charged centres may become greater or less with rising temperature (and *vice versa*) according to the balance of these two opposing tendencies.

The water held in these two ways round uncharged and charged centres in a protein molecule can be called "bound water." The equilibrium is not static but dynamic, that is to say at equilibrium the number of water molecules bound at a centre will be constant though the individuals will be different. The bound water per molecule will be at its maximum when the protein molecules exist freely in solution, as occurs in protein sols. Where the molecules begin to be packed together into systems, as in gels or tissues, some of the centres of hydration become involved in cross-linkages which form between one molecule and another and no longer retain their water (Jordan Lloyd, 1933 a). The less concentrated the structure, therefore, the greater the amount of bound water. It is important to realise how tenaciously proteins hold on to their bound water; pressures amounting to hundreds of atmospheres are unable to drive it completely from the system—yet it can, of course, be removed by evaporation, though the last traces will only be yielded up very slowly to an absolutely dry atmosphere.

An example of the tenacity with which proteins hold bound water is found by balancing these forces against the forces of crystallisation of water itself. These come into play as soon as the temperature of the system falls below 0° C. Moran (1926, 1932) has studied the crystallisation of water from gelatin jellies at low temperatures (see also Mennie, 1932). He finds that with falling temperature, the distribution of the water between the jelly phase and the ice phase is a strict function of the temperature until a certain temperature is reached, namely – 20° C., and beyond this the gelatin still holds 0·5 gm. of water for every 1·0 gm. of protein and continues to hold it against the forces of crystallisation. At this balance, therefore, the forces holding water molecules to the protein are greater than the forces holding water molecules to other water molecules in the ice crystal. At – 20° C. ice can be melted by a pressure of about 3000 atmospheres. This melting is due to the breaking of the intermolecular bonds by the external pressure. It follows, therefore, that some of the water bound to a protein molecule is held by forces greater than those represented by a pressure of 3000 atmospheres.

(b) *The bound water of tissues and organisms.*

The bound water plays an extremely important part in the actual life processes of living cells. If it is reduced below a certain level, the cell dies. The maintenance of its water is one of the most important problems for a living system and there are innumerable adaptations among plants and animals to preserve the water of the body. In land-living organisms, the problem may be said to become acute and will present itself under two forms, firstly the problem of desiccation by evaporation, secondly the problem of desiccation by freezing.

The living cell is able to preserve the concentration of its bound water at a higher level than occurs in the same system after death. The mechanism for doing this is, like the mechanism of so many vital processes, lodged in the membrane of the living protoplasm. The work of Chambers and Hale (1932) has shown that, by freezing, water can be drawn out of the living protoplasm of amoebae, frog's muscle fibres and the cells from the root tip of the onion. The ice forms on the outer surface of the protoplasm but does not form inside the tissue unless the surface is mechanically damaged or unless the temperature is reduced so far that the surface membrane gives way. Ice then forms within the tissue or, in other words, the forces binding the water to the cell proteins become less than the forces of crystallisation of ice. When this stage is reached, the tissue dies. Until it has been reached the water which has moved out across the cell membrane to form ice can go back again into the system, which will fully recover all its vital activities on thawing. The amount of water which can be drawn out of a tissue without causing permanent injury is remarkable. Moran (1929) has shown that a frog's muscle, containing 20 per cent. of dry matter (chiefly protein) and 80 per cent. water, can be dehydrated by either evaporation or freezing until its weight has fallen to 38 per cent. of the original weight. That is to say, that 1 gm. of muscle protein originally associated with 4 gm. of water can give up 3 of these before the system ceases to be a living system. The whole of this water is not to be regarded as water bound to the muscle proteins. Some of it may be bound to other cell constituents and most of it is probably free water, in the sense that it is neither co-ordinated with a protein molecule at a hydration centre nor held by electrostatic forces round a charged centre (Mennie, 1932). Adolph (1933) states that the whole frog can lose 50 per cent. of its water without damage. Probably it is only when the bound water of the cell proteins starts to be drawn out of the system that irreversible damage, resulting in death, takes place. There is no direct evidence bearing on this suggestion, but it is an interesting fact that both plants and animals which are winter hardy retain their bound water, when in the winter-hardened condition, with forces much greater than those of closely allied species which do not develop winter hardness.

This subject has been studied by Newton and his co-workers on the wheat plant (1924, 1926). Some wheats are winter hardy and some are not. In the un-hardened condition, no differences can be detected between the leaves of the two classes, that is to say, in the press juice from the leaves, protein content, sugar

content, etc., are similar and the pressure required to extract the juices from the leaves are of the same order of magnitude. As the cold weather draws on, a change sets in, the protein content of the leaf juices increases, so does the sugar content, but in the winter-hardy species the pressure required to extract the cell juices from the leaves is definitely greater than that required to press out the cell juices of the winter leaves of non-hardy species. This pressure may be taken as a measure of the force with which the cell colloids hold on to their water and may be called imbibition pressure. If the winter-hardened leaves are killed, say with ether, the imbibition pressure immediately falls to the value of the non-hardy winter leaf and free water drains out of the leaf. The increased tenacity with which the water is held by the proteins of the cell juices is, therefore, a function of the living cell.

Winter-hardened leaves have two other characteristics. Their content of catalase (measured by the usual technique of following the rate of destruction of hydrogen peroxide) is much higher than that of either summer leaves or non-hardy winter leaves (Newton and Brown, 1931). Moreover, although at temperatures above the freezing-point their respiratory rate is not noticeably different from that of non-hardened leaves, yet at temperatures below the freezing-point it is considerably less, and parallel with this it is found that the cell sugars only disappear slowly from winter-hardened leaves (Newton and Anderson, 1931). The story seems to run thus: the high catalase activity of the winter-hardened leaves continually destroys peroxides as these are formed in the cell and thus prevents the oxidation of the cell sugars. The sugars protect the proteins from precipitation by freezing (Newton and Brown, 1931), and thus in some way difficult to understand, enable the proteins to hold on to their bound water with a greatly increased tenacity. The plant is thus protected against death by frost.

In a similar way, drought-resisting plants are characterised by the tenacity with which the cell proteins retain their bound water (Newton and Martin, 1930). A series of grasses collected from every type of habitat, from desert to marsh, showed a decreasing percentage of bound water in passing from drought resistant to non-resistant forms. The osmotic pressure of the plant juices also became less, but the corelation between drought resistance and osmotic pressure was not so precise as between drought resistance and bound water.

Winter hardiness has also been studied in certain insects (Robinson, 1927): *Telea polyphemus*, which is winter hardy; *Callosamia promethea*, which is moderately hardy; and *Sitophilus granarius*, the granary weevil, which is non-hardy. Here again, winter hardiness is shown to be exhibited by those species which have the capacity of increasing the resistance with which they hold on to their bound water. *Telea* and *Callosamia* both gained in bound water as the temperature fell; *Sitophilus* lost in bound water.

It seems highly probable that living organisms are capable of maintaining their bound water at a higher equilibrium than in the same system after death, and in some cases at least, that this capacity is associated with an enzyme mechanism and the properties of the cell surface and that energy is consumed to maintain the level. The condition of the bound water in different tissues or in the same tissue at

different times must, therefore, always be kept in mind in considering the movements of water in organisms. It has already been stated that the maximum capacity for binding water to the protein molecule occurs when the molecule is free and falls as the molecules become packed together and connected with cross-linkages.

II. THE FREE WATER OF PROTEIN SYSTEMS.

(a) *Solvent pressure and the distribution of water.*

Having considered the water-binding properties of the protein molecules, the distribution of free water in a system built up of a number of protein molecules must next be considered. The distribution under all circumstances is controlled by an equilibrium between an active force and a resisting force. The active force causing water to diffuse into a system is the actual pressure of the molecules of the liquid water. This is called for convenience the solvent pressure. It is not to be confused with the vapour pressure of the pure solvent. The vapour pressure and the solvent pressure are related functions depending on the state of the molecules in the system, the greater the activity, the greater both the vapour pressure and the solvent pressure and *vice versa*. The solvent pressure is, however, at all times very much greater than the vapour pressure. This can readily be shown by a simple experiment: if a strip of dry leaf gelatin is suspended in a closed vessel, half in water and half projecting into saturated vapour, the lower half in the water swells rapidly and, however long the experiment lasts, is always more swollen than the upper half in the vapour. Again, gelatin swollen strongly in dilute solutions of acid and alkali contracts considerably and expels water in the form of liquid drops when lifted from the solution into an atmosphere of saturated vapour (Jordan Lloyd, 1920; Buchner, 1929); excised frog's muscles also lose a considerable amount of water in an atmosphere of saturated vapour (Jordan Lloyd, 1915). Whole frogs show the same effect, though this has been attributed to the fact that the temperature of the animal is always slightly above that of its surroundings (Adolph, 1933).

(b) *The influence of the cohesive forces of protein systems and of tissues on the distribution of water.*

The solvent pressure of water is, therefore, the pressure of the water in liquid form. The total solvent pressure of the water also depends on such things as the hydrostatic pressure—increasing the head of pressure of a liquid solvent increases the driving force that causes it to diffuse into a system. Under a constant solvent pressure the amount of water which will actually diffuse into a protein system such as a gelatin jelly or a tissue depends on the strength of the cohesive forces of the jelly or tissue, that is, on their resistance to stretching. If the cross-linkages between the protein molecules are few and weak, much more water will diffuse into the system than if they are many and strong. This shows itself with gelatin jellies, which under the same external conditions swell in inverse ratio to the concentration of the sol from which they were set (Jordan Lloyd, 1931), and even more if different tissues are compared with each other (Jordan Lloyd, 1933 a).

The differences in the cohesive forces of the tissues in different parts of the body of a living organism, whether plant or animal, are in themselves sufficient to ensure gradients of distribution of the water even though this be at a constant pressure throughout. The accumulation of cell sap in the growing point of plants and the continuous leakage of the plasma of the blood into the lymph spaces in animals are both examples of the influence of this semi-mechanical factor on water distribution. This factor has received little, if any, attention in the literature and there is a fresh field of investigation here lying open to the research worker that could not fail to give interesting results. It is quite certain that any cytolysis occurring in tissues must necessarily be followed by an accumulation of fluid from this mechanical factor alone, and conversely any change leading to the formation of stable structures within the cell protoplasm, as in the formation of fibres from fibroblasts, must lead to the loss of fluid from that part. The fibrous tissues of animals, such as hairs and connective tissues, are good examples of this, and the fibrous tissues of plants, particularly plants which become woody through the development of a large amount of fibrous tissues, are also examples of the influence of the cohesive forces of a tissue on its water content. The distribution of the water between the two phases at equal solvent pressure is controlled by the cohesion.

This very simple generalisation is sufficient to explain why in marine invertebrates, which possess no osmotic control, the lowering of the freezing-point is, on the whole, slightly greater for the body fluids of the animals than for the sea water in which they live (Schlieper, 1930). The presence of dissolved substances lowers the solvent pressure of a pure liquid in proportion to the amount present. Assuming for the moment that the surface of the living animal body is permeable only to water and not to salts, in shallow waters the pressure on the body surface from without is simply the solvent pressure of sea water. The pressure on the body surface from within, however, is the solvent pressure of the body fluids plus the elastic pressure of the structures of the animal body. In order to make these two pressures equal, which they must be at equilibrium in animals of this type, the solvent pressure of the water in the body fluids must, therefore, be a little less than the solvent pressure of the water in the sea, a state of affairs that can be brought about by slightly increasing the concentration of dissolved substances in the body fluids, a condition which can probably be secured by the influence of the proteins on the ionic distribution.

The distribution of the free water in an organism will therefore be controlled by the cohesive forces of the protoplasm as long as the solvent pressure of the water is kept uniform. What happens, however, if the solvent pressure be varied and what causes solvent pressure to vary?

(c) The influence of dissolved substances on solvent pressure. Membrane equilibria and the properties of living membranes.

The solvent pressure depends on the activity of the solvent molecules. Although "activity" is, strictly speaking, a mathematically defined property, a good working idea of its meaning may be obtained by interpreting the term quite literally—the

molecules, though bound together by cohesive forces, are yet free to move through the body of fluid—each molecule has kinetic energy and the activity of the solvent as a whole is the sum of the activities of the individual molecules. The solvent molecules, therefore, exert a pressure, proportional to their activities, which causes them to diffuse. Now, however, introduce into the solvent some soluble substance. It is to-day well recognised that substances are only soluble in a solvent if they become associated with it. The dissolving of any substance in a solvent, therefore, stays some of the solvent molecules in their courses since they become anchored to the solute. This is particularly striking with electrolytes where each ion anchors a shell of water molecules round itself by electrostatic forces. The result is obviously that these bound molecules cease to be active and the activity of the solvent as a whole falls, hence its diffusion pressure is reduced. It is obvious what must follow if two spaces containing solvent, separated by a membrane through which solvent molecules can pass, are brought into contact with each other. As long as the solvent pressure on both sides is the same, although molecules may cross in both directions, the net effect is the same as if no molecules passed at all. Now, however, try reducing the activity of the molecules on one side of the membrane by dissolving some substance in the solvent. Immediately the solvent pressure falls and fluid begins to flow in across the membrane.

The osmotic pressure of any solution, measured in the ordinary way in an osmometer, is an inverse measure of the solvent pressure. What is actually measured by the osmometer is the extent to which the solvent pressure of the solution has decreased below the solvent pressure of the pure solvent. All dissolved substances, whatever the size and nature of their molecules, reduce the solvent pressure of water. Their influence on the distribution of water across a membrane separating two aqueous phases depends partly on their concentration and partly on their size relative to the size of the pores in the membrane and partly on their chemical nature. If the molecules or ions can cross the membrane, then like the water molecules they will do so and distribute themselves so as to give an equal diffusion pressure and an equal solvent pressure on both sides. Small ions, like those of dissolved salts, can easily cross the ordinary membranes of the laboratory, made of parchment, cellophane or collodion. They cannot cross the so-called semi-permeable membranes formed from colloidal precipitates. They cannot, in general, cross the protoplasmic surface of a living cell with any ease (Lucke and McCutcheon, 1932). Many neutral or unionised molecules, however, readily cross the surface (see, however, Höber and Orskov, 1933). A striking instance of this is found in the comparative toxicity of strong and weak acids and strong and weak bases. Strong acids and bases are completely ionised in dilute solution; weak acids and bases are only partially ionised. Moulds can grow when sulphuric acid in the outer medium gives a pH of < 1, but are killed when acetic acid in the outer medium gives a pH of 3·5 (Pleass, 1933). This is because the ionised sulphuric acid cannot penetrate the living cell, whereas the unionised fraction of the acetic acid can do so, and once within the cell will become partially ionised and kill the protoplasm by virtue of its acidity. In the same way, sea-urchin eggs in sea water can resist the

penetration of sodium hydroxide but are rapidly penetrated and killed by ammonia (Harvey, 1911). Of course, strong acids and bases can, if sufficiently concentrated, destroy the cell surface and penetrate the dead cell.

The free water in an organism might, therefore, be expected to show a relation to the solvent pressure or osmotic pressure of the water in which it lives, particularly when this solvent or osmotic pressure is due to dissolved electrolytes, and certainly, in a general way, it frequently does so. Many useful data on this point have recently been collected by Schlieper (1930). The relation between the osmotic pressure of the blood and the outer fluid is, however, only a very general one and most organisms live in a state of osmotic inequality with their surroundings, a condition which means either covering the body with an impermeable membrane or expending energy on maintaining the inequality. The latter course is the one usually chosen. Adolph (1933) has demonstrated, for instance, that water passes into the frog living in fresh water continually through the skin and is excreted through the kidney. Keys (1933) has shown that the eel in fresh water takes in water through the skin which it excretes through the kidneys; in salt water it takes in water by swallowing it and absorbing it through the walls of the gut.

Although it has been stated that in general the outer surface of living organisms is not readily permeable to ions, all organisms have special organs which are permeable though sometimes only in one direction. The root hairs of plants are permeable to the ions of salts and practically all animals have some organ, such as the kidney, through which salts can diffuse. Wigglesworth (1933 a, b) has recently demonstrated that the anal gills of mosquito larvae are permeable to water and salts while the rest of the animal is impermeable. The surfaces of some of the tissues internal to the body, such as the surface dividing blood capillaries from tissue cells are also, to some extent, permeable to electrolytes. In many cases, however, it seems possible that the passage of dissolved substances across the cell membrane is due to an active secretion rather than a passive diffusion (Höber, 1930, 1932; Lueken, 1932; Whitehouse, Hancock and Haldane, 1929).

So far nothing has been said as to the influence of the proteins of the cell on the distribution of the free water. At the pH found in living tissues most proteins carry an excess of negative charges. The presence of a large colloidal ion in a system influences the distribution of the small ions (Donnan, 1911, 1932; Donnan and Guggenheim, 1932). If introduced on one side of a membrane, it tends to retain a small ion of opposite sign in its neighbourhood, and owing to its own incapacity to cross the membrane, will lead to a greater concentration of ions on the one side and hence a greater reduction of solvent pressure. Obviously, therefore, any system containing proteins carrying an excess of either positive or negative charges will tend to absorb water. The effect of the colloidal ion, however, is greatest when the ratio of concentration of colloid to concentration of electrolyte is high. At the concentration of electrolytes found in protoplasm, the effect of the protein in influencing the distribution of free water within and through the system may not be as great as has sometimes been assumed (Jordan Lloyd and Pleass, 1927).

III. THE MOVEMENTS OF WATER IN LIVING ORGANISMS.

The movements of water in living organisms are controlled by a very complicated equilibrium. Firstly, there are the mechanisms for taking water into the organism; secondly, the mechanisms for distributing it through the organism; thirdly, the mechanisms for retaining as much as is required; and fourthly, the mechanisms for getting rid of what is not required.

The most important chemical mechanism for taking water into a system is undoubtedly the osmotic pressure or, to express it somewhat differently, the reduction of solvent pressure of the pure solvent brought about by the presence in the cell fluids of dissolved substances, particularly salts of the type of sodium chloride. Plants take in water through their external surfaces, notably in the higher plants through the root hairs. Animals take in water in some cases by diffusion across the outer surface, as in the frog and the eel in fresh water, in others by swallowing it, as in the eel in salt water and with all land animals, but even then it has to get across the internal surface of the gut wall before it is really of any use. It also must be remembered that the ordinary metabolic processes produce water. Both plants and animals require energy for growth and other vital activities. Plants can obtain their energy by direct absorption from sunlight; animals obtain it indirectly by the combustion of organic compounds with the oxygen of the air or dissolved air, with the production of carbon dioxide, urea or other nitrogen-containing bodies, and water, all of which must be eliminated.

The most important mechanism for distributing water through the system is the solvent pressure of the water itself, which causes it to diffuse through every tissue that is permeable; coupled with this is the structural system of vessels which convey the water in the higher terrestrial plants and in animals. In the higher plants the force drawing the sap through the vessels is due to evaporation from the leaves plus the cohesive force of the water which holds the columns of water intact. In animals, there is, of course, the pumping action of the heart, which leads to a true circulation of a large percentage of the water and in this way promotes an economy in its use. Of very great importance in controlling the distribution of water which is circulating in an anatomically closed system is the permeability of the vessel walls. The more these leak, of course, the more goes through under the same head of pressure. One of the characteristics of living animals is the control exercised by the body over the permeability of living membranes. In animals this is very often brought about through the action of the nervous system as in the case of the small arterioles and the capillaries. The contraction of these in any part cuts off the water supply; the expansion allows the water of the blood to come into contact with leaky vessels through which the plasma oozes to form the lymph. Besides this it must be remembered that the cohesion of the protoplasm in the different tissues controls the amount of water which can get into them and the osmotic pressure is a factor in the forces drawing water in.

The most important factor for retaining water in a system is undoubtedly the mechanism by which proteins and other cell colloids bind the water molecules and

hold them firmly, making a reservoir of water that can be called on if necessary in adverse circumstances. Dissolved salts or sugars or other crystalloid molecules also act to hold water in the system, though their value from this point of view appears to be less than that of the colloids. Both the imbibitional level and the osmotic level maintained in the living organism are frequently greater than those of a non-living system. The immediate mechanism which enables some plants and animals to retain their water of imbibition against forces leading to desiccation are not fully understood. In winter-hardened wheats, the mechanism appears to be associated with enzyme reactions and therefore probably with the living surfaces of cells. In some animal cells also, the properties of the living surface appear to be involved.

The methods of getting rid of water from a living organism are many and various. It might very well be asked why water has to be eliminated at all; why cannot the balance be preserved simply by controlling the intake?

If it were possible to imagine a living organism so simple that it consisted of nothing but a little cohesive mass of proteins and other cell colloids, the colloidal molecules hanging together and presenting an outer surface permeable to water and dissolved non-ionising crystalloids; if it were possible to imagine such a laboratory model of an organism with food molecules required for energy production and growth and reproduction conveniently drifting in and waste products drifting out, there would be no special need for a stream of water to pass continually through the organism. But it is impossible to imagine the properties of life associated with such a static system. For the living organism to acquire control over its own life processes, there must be a separation between its inner system and the outer system in which it lives. As soon as this happens, then a stream of water must pass continually through the system to carry dissolved food substances in and to carry dissolved excretory products out. In plants, this stream passes in at one end of the machine and out at the other. In animals, which, as a class, are not so much chained to their environment, economy is effected by circulating the water and thus making it do the same work over and over again. In plants, the rôle of the organism in promoting water loss is passive. In aquatic plants it is difficult to know how much water loss occurs, if any. In terrestrial plants, water loss occurs by direct evaporation from the stomata, and adaptations influencing rate of loss are all in the direction of checking evaporation. Plants, as a class, have no definite excretory organs. From the nature of their metabolic processes they do not require them. Animals, however, are different. All animals, from the amoeba upwards, have excretory organs. These excrete not only water and the end-products of metabolism but also salts which the organism needs. The water which passes out of a living organism is, for the most part and under most conditions, the free water of its system. It is, therefore, influenced by forces which reduce its solvent pressure. It has already been mentioned that water and salts pass through the excretory organisms. In marine organisms, the loss of salts is unimportant. In fresh-water or terrestrial animals, the loss of salts may have serious consequences, and in many of these there are special devices for maintaining the salts in the system. Even in marine invertebrates there is some

degree of osmotic control. In the shore crab, *Carcinus maenas*, the osmotic pressures of the blood and urine are the same under different conditions, though both differ from that of the outer medium (Schlieper, 1930). In other animals, such as *Lophius*, a fresh-water teleost fish, the osmotic pressure of the body fluids may be very much greater than that of the outer medium. The kidney is therefore transferring water against the solvent pressure and requires a supply of energy in order to do this work. The excretory system in the human being is a good example of the mechanisms evolved to remove water and control the concentration of salts in the serum. The chief excretory organ is, of course, the kidney, and into the kidney ducts pass the non-colloidal constituents of the blood. The kidney does not, at first sight, appear to have a selective secreting function, since all soluble substances introduced into the body from the food, which are not used in metabolism, pass out in the urine. During the course of years, in laboratories where intermediate metabolism is being studied, the most remarkable range of extraneous organic and inorganic compounds have been taken into the body and, provided they are not toxic, they pass out again through the kidney. The kidney, however, is not a mere passive filter through which small molecules like those of water, urea, sodium chloride, etc., pass by virtue of their diffusion pressure, for apparently both naturally occurring substances, such as uric acid, and unusual substances, such as dyes, may be concentrated in the urine by an active secretory process (Lueken, 1932; Höber and Meirowsky, 1932). Moreover, water and sodium chloride pass back from the kidney into the blood stream.

Any interference with this normal circulation of water and sodium chloride has interesting consequences. Under conditions of muscular activity, where a large amount of heat is produced that has to be dissipated, the kidney is put out of action and the stream of water passing out of the body is diverted so that it now passes through the sweat glands of the skin and by evaporation from the skin surface can carry off the heat (MacKeith, Pembery, Spurrell, etc., 1923). The sweat consists of water, salts, organic acids, urea and numerous other substances with small molecules. Apparently, however, sodium chloride is actively and selectively excreted by the sweat glands, since the heavier the sweat secretion the greater the concentration of sodium chloride (Whitehouse, Hancock and Haldane, 1929). Sweat is like the water used for cooling an internal combustion engine. Its chief function is to carry off heat. In the engine of a car the warm water is cooled in the radiator and returned to the system, but in the engine of a boat fresh water is continually poured through the engine to carry off the heat. In the human engine the warm sweat evaporates or runs off the body and fresh water is continually produced. The sweat glands, however, secrete sodium chloride freely as well as water and return nothing to the blood stream. Heavy or continual sweating may have one of two consequences. Heavy sweating accompanied by large libations of water to quench the resulting thirst may lead to such a heavy loss of sodium chloride from the system that this salt may be washed out of the muscles which then go into a spontaneous tetany or cramp. This matter has been studied especially in connection with miners' cramp, which is caused by men doing heavy work in

hot underground places, getting thirsty and drinking large quantities of water (Whitehouse, Hancock and Haldane, 1929). It has been shown that it can be entirely prevented by adding a trace of common salt to the drinking water. Another consequence of continual sweating is that it leads to the formation of stones in the urinary tracts. One of the functions of the sodium chloride in the urine is that it tends to keep in solution difficultly soluble salts, such as calcium urate, phosphate or oxalate, which in the blood have been prevented from crystallising by the protective action of the blood proteins. These salts pass into the kidney ducts in solution, and generally enough sodium chloride is present to keep them in the dissolved condition as long as the urine is kept warm in the body. When, however, the sodium chloride level is reduced by loss through the skin, these calcium salts may actually crystallise out, even at body temperature, forming concretions or stones. It is a recognised clinical fact that urinary calculi are comparatively rare in temperate climates and common in the tropics, both among Europeans and natives. They also occur in conjunction with a rare disease of the parathyroids which causes a drain of calcium away from the bones into the blood stream; here there is a normal sodium chloride level with a raised calcium level, and though the calcium salts are in solution in the blood, they precipitate out in the kidney ducts. In chronic septic conditions, where there is a large loss of sodium chloride in the pus, urinary calculi also sometimes occur[1].

IV. THE REGULATION OF THE WATER CONTENT OF LIVING ORGANISMS.

These instances are quoted to illustrate the fact, which has been dealt with very fully by Pantin (1931), that in considering the composition of the body fluids in relation to the outer medium it is important to realise that not only the solvent pressure of the water, that is to say the generalised osmotic pressure, is of importance but also the concentration of each separate electrolyte dissolved in the water. Living organisms have a remarkable capacity for controlling the equilibrium level of their water and the dissolved substances. They can, to a large extent, control the osmotic level of the body fluids. This has been shown in the work of Schlieper (1930) and in that of Beadle (1931). *Gunda ulvae*, a small planarium worm found in brackish water, can lead an apparently normal existence in waters varying in salinity from 3 to 44 gm. per litre (Jordan Lloyd, 1914). Many animals can control the concentration level of the sodium chloride, an example of which occurs in the common eel. These fish are bred in the sea but ascend the rivers, where they live the greater part of their lives. In this habitat the sodium content of their blood is higher than that of the fresh water, osmotic control taking place in the kidneys, but when they return to the sea they maintain a sodium chloride level below that of sea water, secreting sodium chloride by special secretory cells in the gills (Keys and Willmer, 1932; Bateman and Keys, 1932; Keys, 1933).

[1] I am indebted for the information in this paragraph to the interest of my friend Mr Cecil Rowntree.

Living organisms, therefore, can to some extent control the osmotic level of their body fluids and in this way regulate the free water of their systems. They can also regulate the bound water. And they can regulate to a large extent evaporation or diffusion of water through the skin. Whitehouse, Hancock and Haldane (1932) have shown that in man there is a diffusion of water vapour through the skin itself as distinct from secretion through the sweat glands. Adolph (1933) has shown in the frog, at any rate, that the skin in the living animal acts as a barrier against water loss, both when the frog is in water and when it is in air. In water, water passes into the frog through the skin but it only passes out through the kidneys. The skin of the normal living frog is, therefore, very much more permeable in one direction than the other. If, however, the frog is pithed, the characteristic of the skin is lost; it now becomes permeable to water and salts in both directions and the permeability to water inwards is greatly increased. The permeability is, therefore, regulated in life by some mechanism which is controlled by the central nervous system. Certainly, water loss by evaporation is under some controlling mechanism in the living organism. Some interesting work has been carried out by Mellanby (1932) on the fasting mealworm. These insects can live for weeks without food. They consume their fat stores which are oxidised, yielding carbon dioxide and water. The ratio of fat used to water produced shows that the weight of carbon oxidised to carbon dioxide and expired is nearly equal to the weight of water produced, so that any change in weight may be fairly attributed to loss or gain of water. The mealworm's body contains, of course, plenty of water besides that produced by its metabolism. Under fasting conditions, the loss of water from the worms goes on at a rate which is inversely proportional to the humidity of the air, but that this is more than a mere physico-chemical process is shown by the fact that the metabolic rate is controlled by the rate of water loss. The mealworm in some way is able to control its metabolism, so that its ratio of dry matter to water is not influenced through the loss of water by evaporation. This it can do only within certain limits of temperature and humidity. If the temperature rises too high, the metabolic rate increases, and in moist atmospheres the water lost by evaporation is less than that produced by metabolism and the creature becomes dropsical. The mealworm is the larva of a tenebrionid beetle and is adapted to hot, dry climates. Under such conditions it can, like the cacti, conserve its water (Newton and Martin, 1930). Under hot, moist conditions, its metabolism gets out of hand and it may get too much water. It is interesting to realise that the bed bug, which generally lives in a moist atmosphere, may be losing water by evaporation in an atmosphere from which mealworms may be taking up water.

V. OXYGEN CONSUMPTION AND THE CONTROL OF THE WATER CONTENT.

The living organism can, therefore, in various ways control the equilibrium levels of its free and bound water. It can only do so, however, by the expenditure of energy. Fox and Simmonds (1933) have shown that certain species of fresh-water animals have a higher respiratory rate than closely allied marine species,

and the implication of this work is that the greater oxygen consumption is called for in response to the demand for energy to do the necessary work to maintain the solvent pressure of the body fluids continually at a lower level than that of the outer water. To keep water from flowing into the organism under these circumstances it must, as it were, be continually pumped back, necessitating the consumption of energy. Beadle (1931) has also shown that in transferring the polychaete worm *Nereis diversicolor* from salt to brackish water there is an increase in weight due to water passing into the worm and, in addition, an increase in oxygen consumption, particularly in the early stages when the worms tend to get swollen by water. Later on, when the animal has adapted its metabolism to the new surroundings, most of the extra water is got rid of and the respiratory rate goes down, though it remains always above the former level in salt water. When the nearly related species *N. cultrifera* is put from sea water into brackish water its weight increases by water absorption until it dies, while the respiratory rate does not increase. Beadle has shown further that with the planarian worm *Gunda ulvae*, which can live in both fresh and salt water, the respiratory rate is inversely proportional to the osmotic pressure of the outer medium. This supports the suggestion that where the solvent pressure of the outer medium is high, as in fresh water, energy is consumed in some mechanism which prevents the penetration of water into the body of the worm. In most cases, nothing is known of the seat of the mechanism controlling the equilibrium of free water, but the observations both of Jordan Lloyd (1914) and of Beadle have suggested that in *G. ulvae* it is localised in the cells lining the wall of the gut.

VI. WATER AND ADAPTATION TO THE ENVIRONMENT.

The successful evolution of the innumerable plant and animal species which survive in all types of environment is generally held to be based on the capacity of the species to adapt itself to the environment. This capacity of adaptation can be shown to depend very largely on the capacity of the organism to resist the environment. This is particularly clearly shown in considering the relation of water to the salts and proteins in a living system. Winter-hardy species are winter hardy because they can resist the desiccating effects of low temperature by changing their physico-chemical equilibria and increasing the forces with which the bound water of their proteins is held; desert-living plants and insects are also drought resistant because they have the capacity of binding water to their cell colloids, generally cell proteins, by very strong forces.

This bound water probably serves two functions. In the first place, as long as it is held to the protein molecule this is probably protected against denaturation. It is known that desiccation by evaporation converts many proteins into a denatured or less soluble form; similarly exposure to low temperatures causes precipitation and denaturation of plant proteins (Newton and Brown, 1931). This precipitation at low temperatures has also been described for the proteins of animal tissues, but has been shown in this instance to be due to a progressive change in the pH of the tissues, which brings about precipitation at the concentration of salts present

(Finn, 1932). The bound water probably protects the proteins of the living cell from adverse circumstances and preserves the molecular organisation of the cell from the disruption which would occur through extreme drying or the formation of ice crystals. The bound water also probably forms a reserve supply to maintain the minimum supply of free water which is essential for the metabolic processes to continue. The mechanism of conserving the bound water against adverse circumstances is a property of the living cell and requires the consumption of energy.

Free water may be defined as water in which the molecules are free to move by virtue of their kinetic energy. Free water will always occur in protein systems in the spaces between the protein molecules. All the chemical processes of life go on in the free water. It is, therefore, of greatest importance to the organism to be able to regulate its supply of free water. Free water reaches all the cells of an organism by its solvent pressure or the pressure of the molecules of the liquid solvent. This is influenced by substances dissolved in the water, particularly electrolytes, since the membranes of living cells are, on the whole, impermeable to ions. The maintenance of the free water at the proper level appropriate to the species is, therefore, closely bound up with the level of the different dissolved salts. Here again, as with bound water, the capacity of certain species to survive in different types of environment is shown to depend on the capacity of the species to resist the environment.

VII. SUMMARY.

The movements of water in living organisms are, in the first place, founded upon the properties of the colloids and crystalloids present in the body. The bound water of the organism is held by the proteins and other cell colloids either by co-ordinated links to definite chemical groupings or by electrostatic forces round charged centres. The free water is distributed through the organism by the ordinary processes of diffusion depending on solvent pressure. Both bound and free water are controlled in their distribution by the cohesive forces between the protein molecules in the different tissues. No sharp distinction can, however, be drawn between "bound" and "free" water, any more than between "colloids" and "crystalloids." The distribution of both bound and free water between an organism and its environment is controlled in the first place by the ordinary physical laws of evaporation, diffusion and so on. It has, however, been repeatedly shown that living organisms can, by the expenditure of energy, maintain inequalities in the distribution level of both water and dissolved salts between the body fluids and the environment. This capacity to resist the environment is one of the most important characteristics of living matter.

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